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Selective cytotoxicity of squamocin on T24 bladder cancer cells at the S-phase via a Bax-, Bad-, and caspase-3-related pathways

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Abstract

Annonaceous acetogenins are a group of potential anti-neoplastic agents isolated from Annonaceae plants. We purified squamocin, a cytotoxic bis-tetrahydrofuran acetogenin, from the seeds of *Annona reticulata* and analyzed its biologic effects on cancer cells. We showed that squamocin was cytotoxic to all the cancer lines tested. Furthermore, squamocin arrested T24 bladder cancer cells at the G1 phase and caused a selective cytotoxicity on S-phase-enriched T24 cells. It induced the expression of Bax and Bad pro-apoptotic genes, enhanced caspase-3 activity, cleaved the functional protein of PARP and caused cell apoptosis. These results suggest that squamocin is a potentially promising anticancer compound.

Keywords: Squamocin; *Annona reticulata*; Acetogenins; Bladder cancer; Apoptosis; Cell cycle

Introduction

Annonaceous acetogenins are a group of potential anti-neoplastic agents isolated from Annonaceae plants (Alali et al., 1999). Recently, annonaceous acetogenins have emerged as potentially promising anticancer drugs for multidrug resistant (MDR) cancers (Oberlies et al., 1997). Structurally, they belong to a series of C-35/C-37 natural products, which possess a terminal γ-lactone ring and a terminal aliphatic side chain connected with some oxygen-bearing moieties, such as zero to three tetrahydrofuran (THF) and/or tetrahydropyran (THP) rings and several hydroxyl groups (Alali et al., 1999). Accumulating data demonstrates that annonaceous acetogenins have antitumor, parasiticidal, pesticidal, antimicrobial and immunosuppressive activities (Alali et al., 1999). The cytotoxicity of annonaceous acetogenins is mediated, at least partly, by the depletion of ATP levels via the inhibition of reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport system (Degli et al., 1994; Hollingworth et al., 1994; Wolvetang et al., 1994; Zafra-Polo et al., 1996). Furthermore, they also inhibit ubiquinone-linked NADH oxidase that is constitutively expressed in the cell membrane of cancer cells, but only transiently expressed in that of normal cells (Morre et al., 1995).

Squamocin, an annonaceous acetogenin with adjacent bis-tetrahydrofuran rings and a free hydroxyl group at the terminal aliphatic side chain (Fig. 1), is isolated from plants of various genera, including *Annona*, *Asimina*, *Goniothalamus*, *Rollinia*, *Uvaria* and *Xylopia* as a major component (Barnes et al., 1995; Chang et al., 1998; Padmaja et al., 1993). To explore the possible application of Formosan annonaceous plants to cancer treatment, we purified various acetogenins, including squamocin from *Annona reticulata* (Chang et al., 1998), and analyzed their biological effects. Using the T24 bladder transitional carcinoma cell line as a model, we showed that squamocin arrested T24 cells at the G1 phase and caused apoptotic cell

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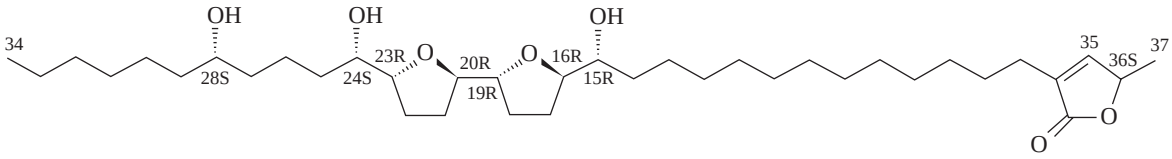


Fig. 1. The chemical structure of squamocin, which is an Annonaceous acetogenin with adjacent bis-tetrahydrofuran rings and a free hydroxyl group at the terminal aliphatic side chain.

death through inducing the expression of pro-apoptotic proteins Bax, Bad and caspase-3.

Materials and methods

Reagents and cell culture

Squamocin was isolated from the seeds of *A. reticulata* (Chang et al., 1998), dissolved in dimethyl sulfoxide, and added to the cultured cells at a 1 to 1000 dilution. To analyze the cytotoxicity of squamocin on various cancer cell lines: bladder cancer cell line (T24), ovarian cancer cell lines (PA-1 and SKOV3), cervical cancer cell lines (HeLa and HeLa S3), breast cancer cell line (MCF7) and skin cancer cell line (BCC-1). T24, SKOV3 and MCF7 cancer cells were grown in Dulbecco modified Eagle medium (DMEM)-F12 supplemented with 10% fetal bovine serum, penicillin, streptomycin and amphotericin B. PA-1, HeLa, HeLa S3 and BCC-1 were grown in Dulbecco modified Eagle medium (DMEM). T24 cells in various cell cycle phases were retrieved using density arrest (Chen et al., 1989), and the M-phase-enriched cells were retrieved by treating the T24 cells (32 h released from density arrest) with 0.4 μg/mL nocodazole for 10 h (Yuan et al., 2002). The cell cycle distribution of T24 cells was analyzed using the EPICS flow cytometer (Beckman Coulter, Fullerton, CA, USA) (Yuan et al., 2002).

Cell proliferation assay

The tetrazolium salt XTT (sodium3-1-(phenylamino-carbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) is metabolized to a formazan dye only by viable cells, and therefore, is utilized in colorimetric assays for the determination of cell proliferation and viability (Scudiero et al., 1988). The cells were plated at a density of 5000 cells/well in 96-well microtiter plates the day before squamocin treatment. After squamocin treatment for 3 days, the cells were subjected to the XTT colorimetric cell proliferation assay, according to the manufacturer's (Roche Molecular Biochemicals, Mannheim, Germany) instructions, to determine the cytotoxicity of squamocin on cancer cells.

Table 1
Cytotoxicity[a] of squamocin on various cancer cell lines, determined by XTT cell proliferation assay

	Cell line	EC50 (μg/mL)[b]
Ovarian cancer cell	PA-1	0.422
	SKOV3	0.421
Cervical cancer cell	HeLa	0.47
	HeLa S3	0.39
Breast cancer cell	MCF7	0.434
Bladder cancer cell	T24	0.233
Skin cancer cell	BCC-1	0.389

[a]For significant activity of pure compounds, an EC_{50} value $\leqq$ 4.0 μg/mL is required (the standard is regulated for NCI) (Yuan et al., 2002). [b]EC_{50} is defined as a 50% reduction of absorbance at a wavelength of 492 nm, with a reference wavelength of 690 nm. Different cancer cell lines were plated into 96-well plates at a density of 5×10^3 cells per well the day before squamocin treatment. Then, the cells were incubated with squamocin at different concentrations for 72 h, before proceeding with the XTT assay.

Cell survival assay

T24 cells were grown in the 6-cm petri dishes and treated with squamocin at different dosages (0, 1, 10 μg/mL) for 24 h. The morphological changes of cells were observed and photographed under an inverted microscope (TC100, Nikon, Minneapolis, Minnesota, USA).

Fluorescence-activated cell sorting analysis

T24 cells (2×10^5 cells/35-mm dish) were treated with squamocin at different dosages for 24 h, harvested by centrifugation, washed with ice-cold phosphate buffered saline, and then resuspended with ice-cold 70% ethanol for 1 h. The ethanol-treated cells were centrifuged, stained with 40 μg/mL propidium iodide for 30 min, and then the DNA content was determined using an EPICS flow cytometer (Beckman Coulter, Oakley, California, USA).

Immunofluorescent staining

T24 cells were grown in 24-well plates, treated with squamocin at various dosages for 24 h and then labeled with 10 μM bromodeoxyuridine (BrdU) for 3 h, according to

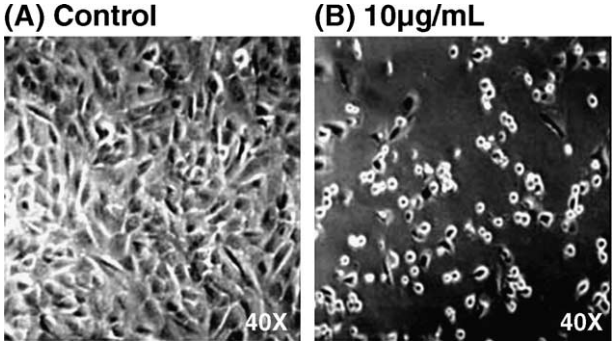


Fig. 2. T24 cells were treated with 10 μg/mL squamocin for 24 h, and the morphological changes were observed and photographed under an inverted microscope.

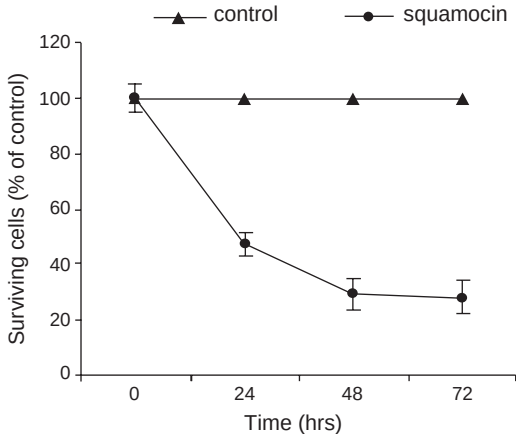


Fig. 3. The cell survival assay shows the percentage of surviving T24 cells after squamocin treatment. T24 cells were treated with 1 μg/mL squamocin for 24, 48, or 72 h, and the numbers of surviving cells are shown as percentages of the untreated control cells. The experiment was repeated three times and the data are presented as means ± SDs. Significant differences were observed between the squamocin-treated group and the untreated group at 24, 48, and 72 h, as determined by Student's *t*-test.

previously published methods (Yuan et al., 1999a). For immunostaining, mouse anti-BrdU monoclonal antibody (Becton-Dickinson) and Texas Red-conjugated goat anti-mouse immunoglobulin G (Jackson Immunochemicals, West Baltimore Pike, West Grove, PA, USA) were used as the primary and secondary antibodies, respectively.

Immunoblotting analysis

Cells were washed twice with phosphate buffered saline after squamocin treatment and then lysed in EBC buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM β-mercaptoethanol, 50 mM NaF, and 1 mM Na_3VO_4), followed by centrifugation. The supernatant was collected and the total protein was measured using the Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada). A previously published immunoblotting procedure was performed (Yuan et al., 1999b). The proteins were detected using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Results

Squamocin has a significant cytotoxicity on different cancer cell lines

To analyze the cytotoxicity of squamocin on cancer cells, cell lines derived from ovarian cancer (PA-1 and SKOV3), cervical cancer (HeLa and HeLa S3), breast cancer (MCF7), bladder cancer (T24), and skin cancer (BCC-1) were tested. The results showed that squamocin was a broad-spectrum anticancer reagent and was cytotoxic to all the cancer lines tested (Table 1).

The cytotoxic effect of squamocin on T24 bladder cancer cells was further analyzed according to morphologic changes caused by squamocin (Fig. 2) and cell survival (Fig. 3). At the dosage of 10 μg/mL, squamocin induced extensive apoptosis and cell detachment at 24 h after treatment (Fig. 2). Additionally, after squamocin treatment at the dosage of 1 μg/mL for 24, 48, and 72 h individually, only 47.0%, 29.0%, and 28.0%, respectively, of the T24 cells survived, as determined by the cell survival assay, in comparison to untreated controls (Fig. 3).

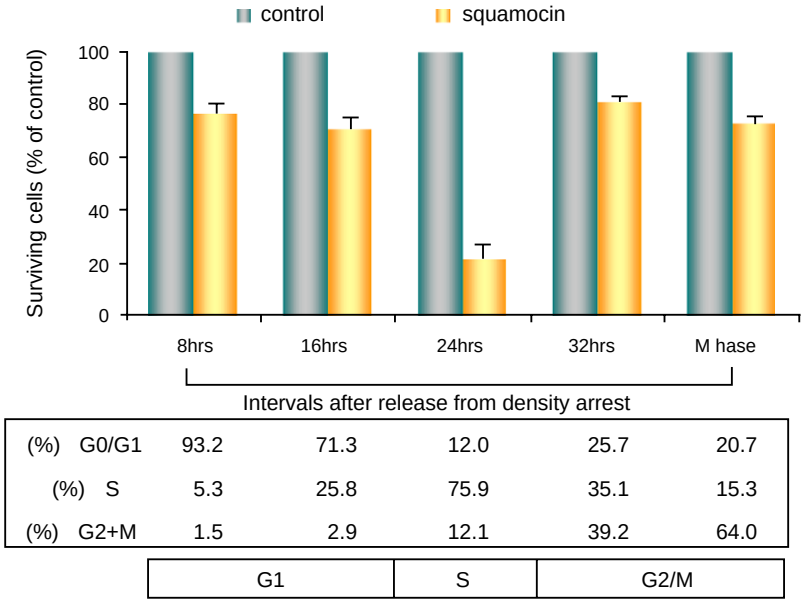


Fig. 4. Cytotoxic effect of squamocin on T24 cells at various cell cycle phases. T24 cells at various cell cycle phases were treated with squamocin at a dosage of 10 μg/mL for 4 h, and then the percentage of surviving cells were determined by cell survival assay. The experiment was repeated three times and the data are presented as means ± SDs. A significant difference ($p < 0.05$) was observed between the squamocin-treated S-phase cells and squamocin-untreated cells at the S-phase, as determined by Student's *t*-test.

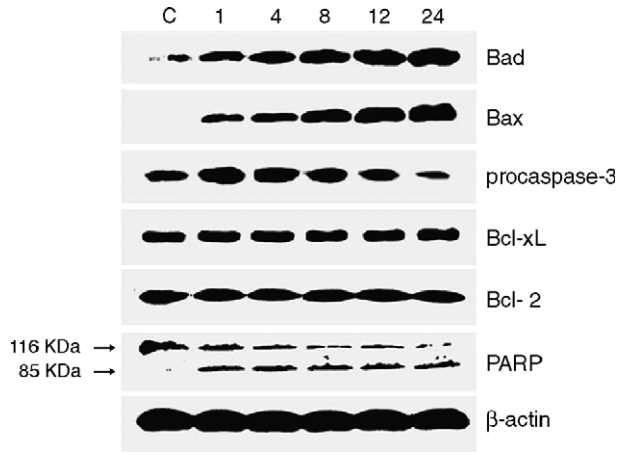


Fig. 5. Expression of the pro-apoptotic and anti-apoptotic genes after squamocin treatment. T24 cells were treated with 10 μg/mL squamocin for different periods and harvested for immunoblotting analysis. C=untreated control; 1, 4, 8, 12, 24-h squamocin treated cells.

Selective cytotoxicity of squamocin on S-phase-enriched T24 bladder cancer cells

We also analyzed cell cycle dependency for the cytotoxicity of squamocin. T24 cells in different cell cycle phases (Methods and Chen et al., 1989) were treated with squamocin at the dosage of 10 μg/mL and the percentages of surviving cells were determined by cell survival assay at 4 h after treatment. Interestingly, S-phase-enriched T24 cells were more vulnerable to squamocin treatment than cells at other phases, with only 21.1% of the cells surviving at 4 h after treatment (Fig. 4).

Pro-apoptotic and anti-apoptotic protein expression after squamocin treatment

The apoptotic pathways for different cytotoxic agents are complex (Huang and Oliff, 2001; Strasser et al., 2000; Johnstone et al., 2002). To determine the detailed mechanism for squamocin-induced cell death, various pro-apoptotic and anti-apoptotic genes were explored for their possible involvement in the cytotoxicity of squamocin. Squamocin significantly enhanced the expression of Bax and Bad, but not Bcl-2 or Bcl-xL, at 1, 4, 8, 12, and 24 h after treatment (Fig. 5). Furthermore, the expression of procaspase-3 was induced and the function of PARP was destroyed by 10 μg/mL of squamocin at 1 h and greater time points via cleavage (Fig. 6).

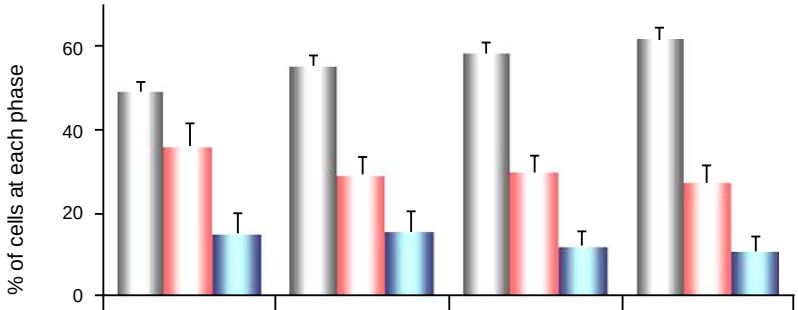
Squamocin inhibited cancer cell proliferation

T24 cells were treated with squamocin at various dosages for 24 h and the cell cycle distribution was determined by flow cytometry. Squamocin arrested T24 cells at the G1 phase in a dosage-dependent manner (Fig. 6). At the dosage of 1 μg/mL, G1 phase cells increased by 12.7% and S- and G2/M-phase cells decreased by 8.6% and 4.1%, respectively. Furthermore, squamocin also inhibited S-phase progression, as determined by the BrdU labeling assay (Fig. 7). After 1 μg/mL squamocin treatment for 24 h, BrdU-positive cells were decreased by 26.3% in comparison to untreated cells.

We also analyzed the expression of p21 and chk2, the cell cycle checkpoint proteins that are involved in G1 and G2/M checkpoint controls, respectively, in T24 cells after squamocin treatment. Neither changes at the expression levels nor band shifting were observed for chk2 after squamocin treatment (Fig. 8). On the other hand, the expression of p21 in T24 cells was enhanced dramatically upon squamocin treatment, although functional p53 was absent in T24 cells (Cooper et al., 1994) (Fig. 8).

Discussion

The major three groups of acetogenins have been reported that the cytotoxic activity of the adjacent bis-THF is better than the nonadjacent bis-THF and monotetrahydrofuran (THF) (Alali et al., 1999; Oberlies et al., 1997). In this study, we showed that squamocin, an adjacent bis-THF (Fig.



	0 ∝g/mL	0.01 ∝g/mL	0.1 ∝g/mL	1 ∝g/mL
G0/G1	48.8	55.2	58.3	61.5
S	36	29.2	29.7	27.4
G2/M	15.2	15.6	12	11.1

Fig. 6. Squamocin arrested T24 cells at G1 phase. Flow cytometric analysis was performed after T24 cells were treated with squamocin at different dosages for 24 h. The experiment was repeated four times and data are presented as means±SDs.

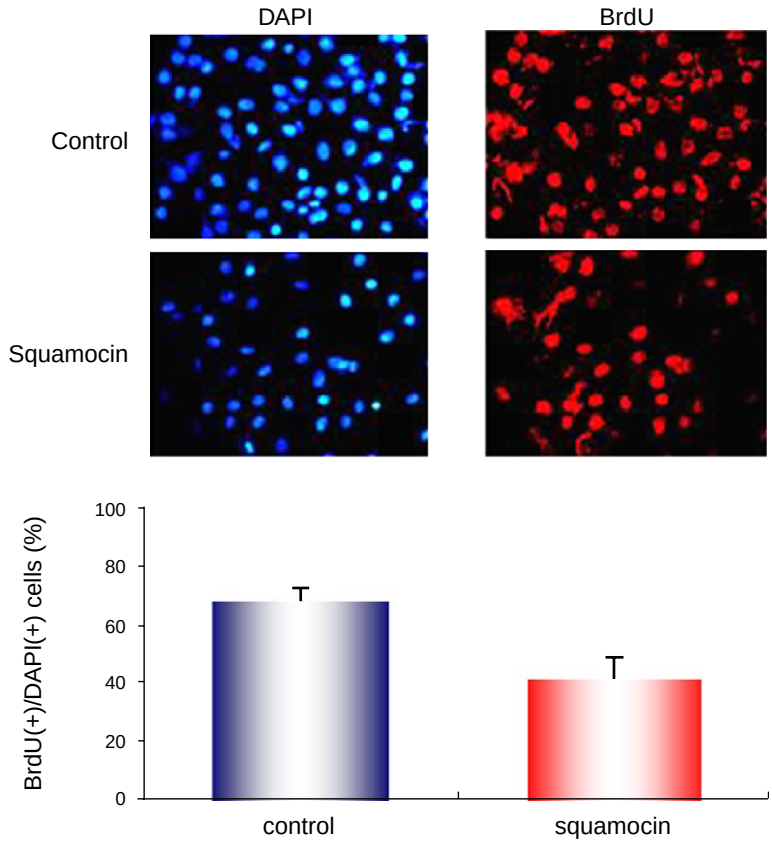


Fig. 7. Effect of squamocin on S-phase progression as determined by BrdU labeling and immunofluorescent staining. T24 cells were treated with 1 μg/mL squamocin for 24 h and then labeled with 10 μM BrdU for 3 h. More than 1000 DAPI-positive cells were counted. The DAPI nuclear staining is shown in blue and BrdU staining is shown in red. The significance of differences between the squamocin-treated group and the untreated group were determined using Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1) had significant cytotoxicity against various cancer cell lines, with an EC_{50} of less than 4 μg/mL that was defined as the minimal requirement for an active pure compound (Geran et al., 1997) (Table 1). Furthermore, we tested the cytotoxicity of squamocin for non-tumorigenic mammary epithelial MCF 10A and the result showed that squamocin had lower cytotoxic activity for MCF 10A than other cancer cell line (EC_{50}=10 μg/mL).

The mechanisms for the biological effects of squamocin on p53-deficient T24 bladder cancer cells were further analyzed and our result showed that the expression of p21 in T24 cells was induced after squamocin treatment, which suggests that squamocin arrests cells at the G1 phase in a p53-independent pathway (Fig. 8).

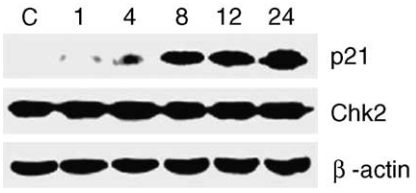


Fig. 8. Expression of cell cycle checkpoint proteins after squamocin treatment. T24 cells were exposed to a dosage of 10 μg/mL squamocin for different durations and subjected to Western blot analysis. C=untreated controls; 1, 4, 8, 12, 24-h squamocin treated cells.

In T24 cells, the expression of Bax and Bad, two pro-apoptotic proteins involved in the mitochondria-mediated cell death pathway, was enhanced by squamocin treatment (Fig. 5). Although procaspase-3 was induced at 1 h and later time points (Fig. 5), the P12 and P17 subunits of caspase-3 did not appear after squamocin treatment in T24 cells. A similar observation was also reported by Seo and Surh, when human promyelocytic leukemia HL-60 cells were treated with eupatilin (Seo and Surh, 2001), and the reason for this observation is currently unclear.

According to a previous study, stress-activated protein kinase (SAPK/JNK) was activated in apoptotic HL-60 cells, after treatment of squamocin (Zhu et al., 2001). However, we were unable to detect the activation of MAPK members including p38, ERK and JNK in T24 cells after squamocin treatment (data not shown). It is presumably possible that squamocin-induced apoptosis in different cancer cells may be through different mechanisms.

Conclusion

Our study demonstrated that squamocin activated p21 and arrested bladder cancer cell growth at the growth-static G1 phase and also caused significant cytotoxicity to cancer cells at the S-phase. These two biological characteristics suggest that squamocin is a promising anticancer agent and worthy of further studies.

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