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Induction of G2/M phase arrest by squamocin in chronic myeloid leukemia (K562) cells

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Abstract

Squamocin is one of the annonaceous acetogenins and has been reported to have anticancer activity. Squamocin was found to inhibit the growth of K562 cells in a time- and dose-dependent manner. Cell cycle analysis showed G2/M phase arrest in K562 cells following 24 h exposure to squamocin. During the G2/M arrest, cyclin-dependent kinase inhibitors (CDKIs), p21 and p27 were increased in a dose-dependent manner. Analysis of the cell cycle regulatory proteins demonstrated that squamocin did not change the steady-state levels of Cdk2, Cdk4, cyclin A, cyclin B1, cyclin D3 and cyclin E, but decreased the protein levels of Cdk1 and Cdc25C. These results suggest that squamocin inhibits the proliferation of K562 cells via G2/M arrest in association with the induction of p21, p27 and the reduction of Cdk1 and Cdc25C kinase activities.

Keywords: Squamocin; G2/M arrest; Cyclin; K562 cells

Introduction

Cell cycle control is the major regulatory mechanism of cell growth (Torres and Horwitz, 1998; Gamet-Payrastre et al., 2000; Murray, 2004). Many cytotoxic agents and/or DNA damaging agents arrest the cell cycle at the G1, S or G2/M phase and then induce apoptotic cell death (Orren et al., 1997; Fujimoto et al., 1999; Gamet-Payrastre et al., 2000). The cell cycle check-point may function to ensure the cells have time for DNA repair (O'Connor et al., 1993; Murray, 2004). In recent years, considerable advances have been made in understanding the roles of cyclins, cyclin-dependent kinases (Cdks), cyclin-dependent kinases inhibitors (CDKIs) in cell cycle progression. This process is regulated by the coordinated action of Cdks in association with their specific regulatory cyclin proteins (Murray, 2004). Primary regulators of G1 progression are the D-type cyclins (D1, D2, D3), cyclin E, and Cdks. Cdk4 and Cdk6 are activated in early G1 by interactions with D-type cyclins, whereas Cdk2 is activated in late G1 by interaction with cyclin E, and at the G1/S boundary and throughout the S phase by interaction with cyclin A. G2 to M phase progression is regulated by a number of the Cdk/cyclin family, especially, activation of the Cdk1/cyclin B1 complex is required for transition from G2 to the M phase of the cell cycle (Lewin, 1990; Doree and Galas, 1994). CDKIs including $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ also contribute to the

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regulation of cell cycle progression by controlling Cdk activity (Sherr, 1996; Coqueret, 2003). Several studies have shown that various cytotoxic drugs can induce G2/M phase accumulation (King et al., 1994; Ling et al., 1998; Sleiman et al., 1998).

In the search for new cancer therapeutics, the herbs being used in traditional medicines for cancer treatment are promising candidates. Squamocin is a bis-tetrahydrofuran *Annonaceous* acetogenin isolated from several genera of the plant family Annonaceae, the structure of which (Fig. 1) is characterized by a long alkyl chain bearing a terminal α, β-unsaturated γ-lactone ring, two tetrahydrofuran rings, and some oxygenated substitutes along the chain. It has been previously reported that this compound could produce potent cytotoxicity against a variety of cells (Fujimoto et al., 1998; Duret et al., 1999; Raynaud et al., 1999; Queiroz et al., 2000). Moreover, it has recently been reported that squamocin could induce apoptosis in HL-60 leukemia cells, and this effect was implicated in the activation of caspase-3 and stress-activated protein kinase (Zhu et al., 2002). To date, however, the molecular mechanism(s) by which squamocin exert their cytotoxic effects is unclear.

To elucidate the mechanism of squamocin, squamocin was used together with K562 cells, a valid model for testing anti-leukemia as well as general anti-tumor agents. We found that treatment with squamocin on K562 cells resulted in G2-growth arrest, presumably involving the concomitant reduction of Cdk1 and Cdc25C and marked upregulation of p21 and p27.

Materials and methods

Chemicals

RPMI 1640 medium, fetal calf serum (FCS), trypan blue, penicillin G and streptomycin were obtained from GIBCO BRL (Gaithersburg, MD). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MD). Antibodies against p21, p27, cyclin A, B1, D3, E, Cdk1, Cdk2, Cdk4, and Cdc25C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Pierce (Rockford, IL). Hybond ECL transfer membrane and ECL Western blotting detection kit were obtained from Amersham Life Science (Buckinghamshire, UK).

Preparation of squamocin solution

Squamocin (Fig. 1) was provided by Professor Yang-Chang Wu, Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan. The structure of this compound has been verified by means of mass spectrometry and spectroscopic techniques (Chen et al., 1999). Squamocin was dissolved in dimethyl sulfoxide (less than 0.01%) and made immediately prior to experiments.

Cell culture

Human leukemia K562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and human purified lymphocytes preparation was obtained from blood as described previously (Su et al., 2003). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Cell growth inhibition assay

The viability of cells was determined by the trypan blue dye exclusion method and assessed by the MTT assay. Exponentially growing cells (1×10^5) were plated in 96-well plates and after 24 h of growth treated with a series of concentrations of squamocin. Incubation was carried out at 37 °C for 72 h. Cells exposed to 0.2% trypan blue were counted in a hemocytometer. MTT solution was added to each well (1.2 mg/ml) and incubated for 4 h. MTT is reduced by the mitochondrial dehydrogenases of viable cells to a purple formazan product. The MTT–formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm in an ELISA plate reader.

Flow cytometric analysis

Controlled and treated cells were harvested, washed in cold PBS, fixed in 70% ethanol and stored at 4 °C. DNA was treated with RNase-A solution (500 unit/ml) at 37 °C for 15 min and stained by propidium iodide (50 μg/ml) in 1.12% sodium citrate at room temperature before analysis. Flow cytometric determination of DNA content was analyzed by COULTER EPICS XL Flow Cytometer (Coulter Corp., Miami, FL, USA). The fractions of the cells in G0/G1, S, and G2/M phase were analyzed using a cell cycle analysis software, Multicycle (Phoenix flow system, San Diego CA, USA).

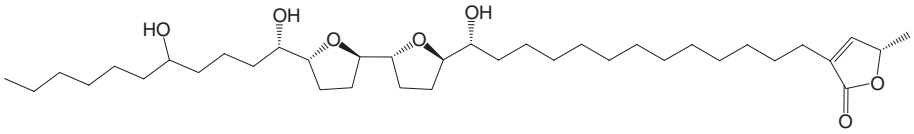

Fig. 1. Chemical structure of squamocin.

Western blot analysis

Cells were washed in PBS, suspended in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and placed on ice for 30 min. After centrifugation at 20,000×*g* for 30 min at 4 °C, the supernatant was collected. The protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Whole lysate (50 μg) was resolved by 12% SDS-PAGE, transferred onto PVDF membranes (Roche) by electroblotting, and probed with anti-p21, -p27, anti-Cdk1, -Cdk2, -Cdk4, anti-cyclin A, -cyclin B1, -cyclin D3, -cyclin E and anti-Cdc25C (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blot was developed by enhanced chemiluminescence.

Results

Inhibition of cell growth

To verify the effect of squamocin on cell growth, K562 cells were treated with increasing concentrations of squamocin or DMSO for 72 h, and cell survival was assessed by MTT assay. As shown in Fig. 2A, survival was inversely correlated with squamocin concentration. Significant loss of viability was detected at 1, 2, 5, 10 and 20 μg/ml squamocin in a dose- and time-dependent manner (Fig. 2A, $P<0.05$). Cell growth inhibition by squamocin was further confirmed by trypan blue dye exclusion method, and the results are shown in Fig. 2B. Proliferation of K562 cells was significantly suppressed in the presence of squamocin in a concentration-dependent manner. Meanwhile, the parallel treatment of squamocin to normal human lymphocytes showed much less strong effect on inhibition of viability (Fig. 2C), indicating that transformed (K562) cells are more susceptible to squamocin. Thus, we chose 1, 2 and 5 μg/ml squamocin to detect changes in molecular events in the following experiments.

Squamocin induced an arrest in G2/M phase in K562 cells

To determine if cell growth inhibition involves cell cycle changes, we examined cell cycle phase distribution by flow cytometry. When cells were treated with various concentrations of squamocin for 24 h, or with 5 μg/ml of squamocin for the indicated time points, a similar level of G2/M phase arrest was observed in a dose- and time-dependent patterns (Fig. 3A). Squamocin treatment induced an accumulation of cells in the G2/M phase of the cell cycle, mainly evident at the concentrations of 1, 2 and 5 μg/ml. For example, 5 μg/ml squamocin treatment for 24 h resulted in an increase in the percentage of cells in the G2/M phase from 13.3 to 33.0%. Concomitant with this increase in the percentage of cells in the G2/M phase was a significant decrease in the percentage of cells in the G0/G1 phase from 36.8 to 21.9%. These results suggested that squamocin inhibited the cellular proliferation of K562 cells via G2/M phase arrest of the cell cycle.

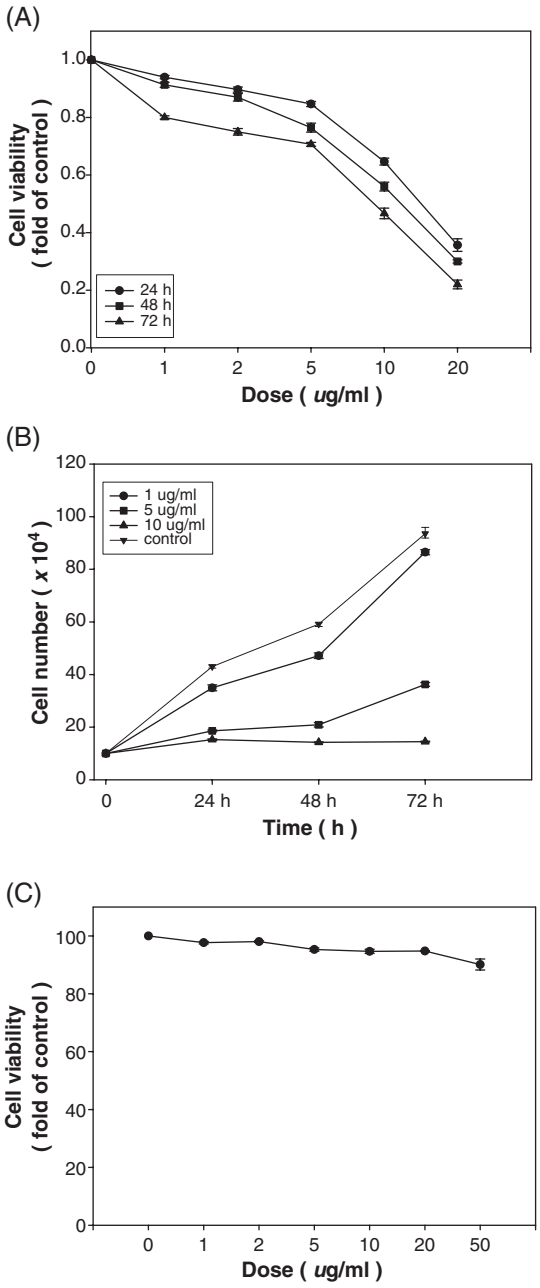

Fig. 2. Effect of squamocin on proliferation of K562 cells and viability of normal lymphocytes. (A) MTT assay of K562 cells, (B) trypan blue dye exclusion assay of K562 cells and (C) MTT assay of the viability of normal lymphocytes. Cells were incubated with a series of indicated concentrations of squamocin for 72 h, and cell survival was determined. The percentage of viable cells was calculated as a ratio of treated to control cells (treated with PBS). Data are mean±SD of three independent experiments.

After cell cycle arrest at the G2/M phase, squamocin-treated cells underwent apoptosis. The sign of apoptosis was indicated by the accumulation of sub-G1 population in K562 cells after squamocin treatment. After 24, 48, and 72 h treatment with indicated concentrations of squamocin, the cells displayed apoptotic/hypodiploid peaks, especially at 72 h with 5 ìg/ml of squamocin (Fig. 3B).

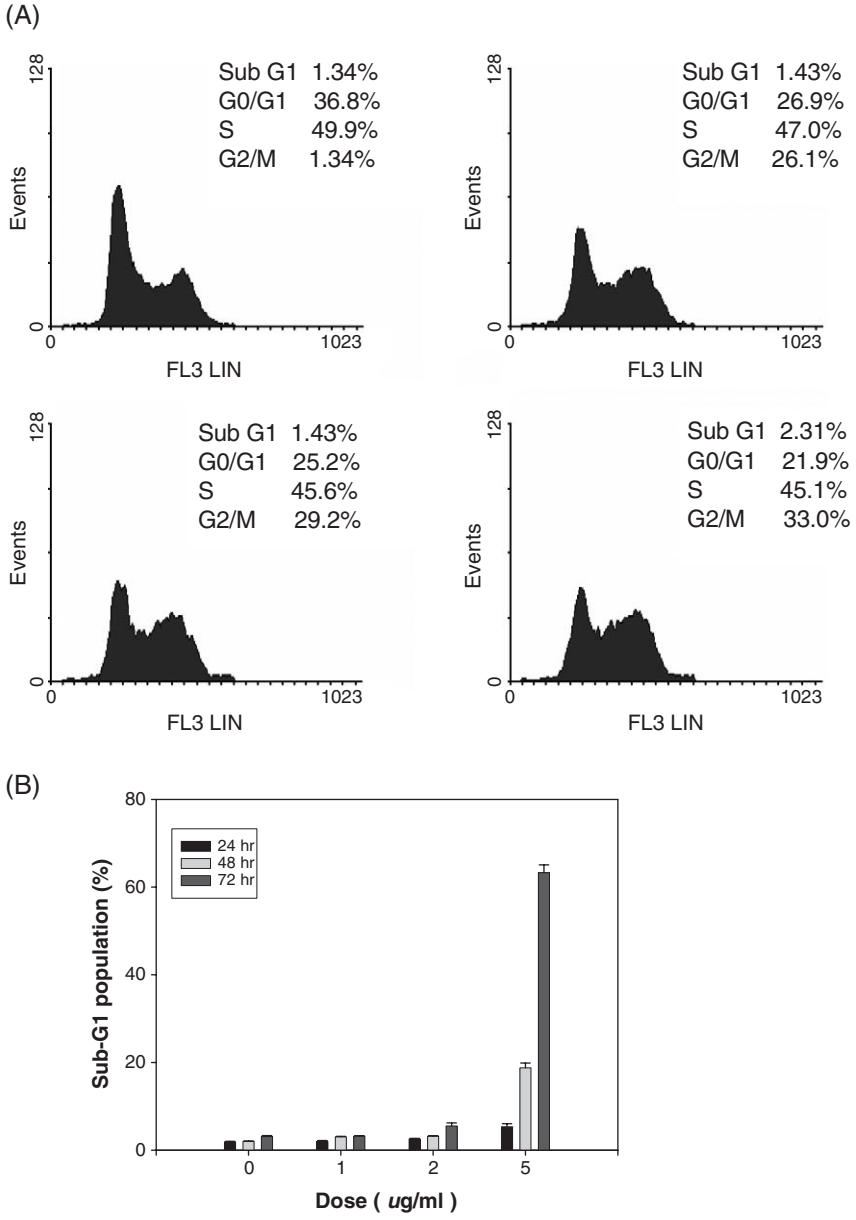

Fig. 3. Flow cytometric analysis of squamocin-treated K562 cells. (A) Accumulation of G2/M phase of cell cycle in squamocin-treated K562 cells. Cells were cultured for 24 h in the absence or in the presence of 1, 2 and 5 μg/ml of squamocin. (B) Sub-G1 population of K562 cells treated with indicated concentrations of squamocin at 24, 48, and 72 h. Cells stained with propidium iodide were subjected to flow cytometric analysis for cell distributions at each phase of cell cycle, as described in Materials and methods.

Effects of squamocin on proteins involved in G2 to M transition

Cyclin-dependent kinase inhibitors (CDKIs) play a key role in controlling cell cycle progression by negatively regulating the Cdks activities at an appropriate time in the cell cycle (Coqueret, 2003). Since squamocin induced G2/M arrest in K562 cells, we wanted to determine the levels of CDKIs, p21 and p27 protein in K562 cells exposed to 5 μg/ml of squamocin. The levels of p21 and p27 proteins were progressively increased in a dose-dependent manner after treatment with squamocin (Fig. 4). In vertebrate cells, the G2/M transition is triggered by regulation of cyclin B1–Cdk1 complex and Cdc25C which promote the breakdown of the nuclear membrane, chromatin condensation, and microtubule spindle formation (King et al., 1994). Therefore, the effect of squamocin on the protein levels of Cdk1, cyclin B1 and Cdc25C was examined in order to learn about the regulation of G2/M phase arrest in K562 cells. Immunoblotting revealed that squamocin treatment resulted in a significant reduction in the protein levels of Cdk1 and Cdc25C but not in cyclin B1 in a concentration-dependent manner (Fig. 4). However, there was no change in the protein levels of cyclin D3, E and A which regulate the G1 progression and G1/S transition (Fig. 4), and their related Cdks (Cdk2 and Cdk4) were also not changed at 24 h of exposure (Fig. 4). These results suggested that squamocin treatment might cause a reduction in kinase activity

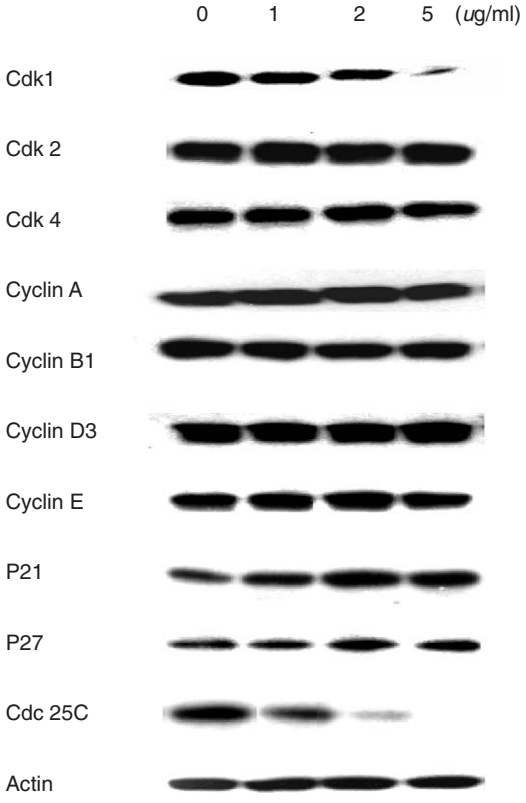

Fig. 4. Immunoblot analysis for the levels of cell cycle regulatory proteins. K562 cells were treated with different concentrations as indicated for 24 h. Total cell lysates were prepared and 50 μg/ml protein was subjected to SDS-PSGE followed by Western blot analysis and chemiluminescent detection. Each antigenic protein was detected by using the respective antibody against Cdk1, Cdk2, Cdk4, cyclin A, cyclin B1, cyclin D3, cyclin E, p21, p27, Cdc25C or β-actin.

of the Cdk1/cyclin B1 complex to elicit G2/M phase cell cycle arrest mainly due to a decline in the protein levels of Cdk1 and Cdc25C.

Discussion

Many anticancer agents and DNA-damaging agents arrest the cell cycle at the G1, S, or G2/M phase and then induce apoptotic cell death (Orren et al., 1997; Fujimoto et al., 1999; Kessel and Luo, 2000). Cell cycle check-points may function to ensure that cells have time for DNA repair, whereas apoptotic cell death may function to eliminate irreparable or unrepaired damaged cells. Squamocin had time- and dose-dependently antiproliferative effect on K562 cells, but the mechanism is poorly understood. The purpose of the present study was to elucidate molecular mechanism of action by which squamocin inhibited proliferation of human leukemia K562 cells. As shown in Fig. 3, squamocin induced a time- and dose-dependent accumulation of K562 cells in the G2/M phase of the cell cycle. G2/M phase accumulation has been observed in cells exposed to DNA damaging agents such as γ-irradiation (Hyun et al., 2002), microtubule-stabilizing agents (Ling et al., 1998), and topoisomerase inhibitors (Lock and Ross, 1990). To the best of our knowledge, this is the first report describing the mechanism of G2/M phase arrest of squamocin on K562 cells.

Our cell cycle analysis revealed a prominent G2/M arrest of K562 cells after their exposure to squamocin. Unlike the p16 family, the p21 family of CDKIs has a broad range of specificity in the cell cycle proteins and is able to inhibit all the G1 cyclin–CDK complexes as well as cyclin B1–Cdk1 complexes (Molinari, 2000; Murray, 2004). In this study, the G2/M phase arrest in K562 cells was associated with a marked upregulation of p21 and p27 proteins, suggesting that the induction of p21 and p27 is an important event by squamocin-induced antiproliferative effect. Additionally, our data suggest that squamocin caused CDKIs upregulation involving p53-independent pathway, as K562 cells lack functional p53.

A number of Cdks have been isolated and shown to regulate the cell cycle event in mammalian cells (Hartwell and Weinert, 1989; Molinari, 2000). Among Cdks that regulate cell cycle progression, Cdk1 kinase is activated primarily in association with cyclin A and B1 in the G2/M phase progression. In this study, we found that Cdk1 protein was decreased in a dose-dependent manner following the treatment with squamocin, in contrast, the protein level of cyclin A and cyclin B1 was not changed (Fig. 4). Although cyclin A and cyclin B1 are known to be involved in G2/M cell cycle progression, the cyclin B1/Cdk1 complex is the primary regulator of transition from G2 to M phase (Doree and Galas, 1994). Thus, our data suggest that cell cycle arrest is mediated by limitation of the supply of Cdk1 to Cdk1/cyclin B complex formation, which is an essential step in regulating passage into mitosis.

In addition, there was no change in the levels of cyclin D3, cyclin E, and their associated Cdks (Cdk2 and Cdk4) which are activated during the G1 progression and G1/S transition. Meanwhile, the Cdk1/cyclin B1 kinase complex is maintained in an inactive state by reversible phosphorylations on tyrosine 15 and threonine 14 of Cdk1 (Molinari, 2000). At the onset of mitosis, both of these residues are dephosphorylated by the Cdc25 family of phosphatases, such as Cdc25B and Cdc25C, and this reaction is believed to be the rate-limiting step for entry into mitosis (Hartwell and Weinert, 1989; Molinari, 2000). We found that squamocin treatment caused a significant reduction in the expression of Cdk1 and Cdc25C in K562 cells (Fig. 4, $P<0.05$). Thus, it is reasonable to postulate that squamocin treatment may cause cell cycle arrest by reducing the activity of Cdk1/cyclin B kinase complex due to down-regulation of protein levels of Cdk1 and Cdc25C. Recently, mitogen-activated protein kinase (MAPK) family of serine/threonine kinases has emerged as an important component of cellular signal transduction (Wada and Penninger, 2004). MAPK family members have been implicated in events such as apoptosis, cell cycle and differentiation. Three MAPK families have been described: the extracellular signal-regulated kinases (ERK), the c-jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and the p38 kinases. In the future, we plan to elucidate interrelationship between these signal pathways.

In summary, squamocin inhibits the proliferation of K562 cells through G2/M phase arrest, which is mediated by down-

regulation of Cdk1 kinase activity in association with induction of p21 and p27 and reduction of Cdc25C. Finally, these results suggest that squamocin may be useful as one of the investigational compounds in the treatment of leukemia.

Acknowledgments

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