Smenospongine, a spongean sesquiterpene aminoquinone, induces erythroid differentiation in K562 cells

Shunji Aoki[a], Dexin Kong[a], Kouhei Matsui[a] and Motomasa Kobayashi[a]

The differentiation of K562 chronic myelogenous leukemia (CML) cells by smenospongine, which is a sesquiterpene aminoquinone isolated from a marine sponge, was examined. Smenospongine increased hemoglobin production in K562 cells at concentrations of 3–15 μM. In addition, flow cytometric analysis of smenospongine-treated K562 cells with FITC-labeled glycophorin A antibody showed an increase of glycophorin A expression, a marker for erythroid differentiation. Cell-cycle analysis showed G_1 arrest in K562 cells after treatment with smenospongine for 24 h. The effect on expression of CIP/KIP family cyclin-dependent kinase inhibitors was investigated by Western blotting analysis and the result showed increased expression of p21, which is known to play an important role in differentiation. Furthermore, smenospongine was also found to inhibit the phosphorylation of Crkl, a substrate of Bcr–Abl tyrosine kinase, which is known as a causative protein of CML. In conclusion, our investigation indicated that smenospongine induced the differentiation of K562 cells into erythroblasts along with cell-cycle arrest at G_1 phase and the mechanism might be attributed to the increased expression of p21.
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Introduction

Anticancer agents play an important role in cancer therapy today. Most anticancer drugs have been developed based on their cytotoxic properties against tumor cells by targeting cellular organelles, which also exist in normal cells. The adverse effect on proliferative normal cells by these drugs has been a serious problem. Therefore, recent research has been focusing on identification of the factors characteristic to carcinogenesis and cancerous proliferation, and exploitation of new anticancer agents selective to cancer cells is ongoing.

Disruption of the checkpoint of the cell cycle is one major cause of carcinogenesis [1]. In order to differentiate into mature cells having an inherent function, normal cells arrest progression of the cell cycle to repair minor damage and induce apoptotic cell death in response to severe damage. In the case of cancer cells, the perturbed checkpoint of the cell cycle allows infinite proliferation at the premature stages. Therefore, chemicals which arrest the cell cycle and repair the function of the checkpoint of the cell cycle are expected to be used as anticancer agents with minimal side-effects. In fact, some clinically observed regression of tumors has been accompanied with histopathological findings which indicate the differentiation of the cancer cells into mature cells [2]. In recent years, all-*trans* retinoic acid (ATRA) has been clinically proven to be effective for the treatment of acute promyelocytic leukemia (APL) as a differentiation inducer [3]. However, the degree of susceptibility of cancer cells against ATRA is not the same and the development of new differentiation inducers against ATRA-non-responsive cancer cells is required.

On the other hand, chronic myelogenous leukemia (CML) arises from chromosomal abnormality in a pluripotent hematopoietic stem cell. Philadelphia translocation (Ph1), t(9;22), has been found in more than 90% of CML [4] and the transcripted Bcr–Abl tyrosine kinase is the major cause of pathogenesis in CML [5]. Imatinib mesylate (Glivec) has been developed as a selective inhibitor of Bcr–Abl and caused apoptosis in CML [6]. Nonetheless, certain CML cells are found to be non-responsive to imatinib mesylate.

As a part of our study of biologically active substances from marine organisms we have been searching for new differentiation-inducing substances for cancer cells. So far, we have found several inducers of cell differentiation from marine sponges: scalarane-type sesterterpenes (PHCs) [7], long-chain acetylene alcohol (lembehyne A) [8] and pyridoacridine alkaloids [9]. We further isolated smenospongine, a sesquiterpene aminoquinone, from an Indonesian marine sponge. Smenospongine

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induced cell differentiation of K562 CML cells [10] into erythroblasts. This paper presents details of the differentiation activity of smenospongine.

Materials and methods

Materials

Gel cassettes (4–20% and 15–25%) were purchased from Daiichi (Tokyo, Japan). DNA-Prep Reagents Kit was from Coulter (Miami, FL). RPMI 1640 medium was from Nissui Pharmaceutical (Tokyo). Polyvinylidene fluoride (PVDF) membrane was from Amersham Pharmacia (Little Chalfont, UK). Other reagents were from Sigma (St Louis, MO) or Wako (Osaka, Japan).

Cell culture

Human CML cell line (K562), which was provided by Riken Cell Bank, was routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml kanamycin and 0.44 mg/ml glutamine at 37°C in a humidified atmosphere containing 5% CO_2.

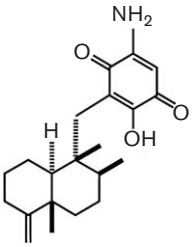
Antibodies

Fluorescein isothiocyanate (FITC)-labeled glycophorin A antibody was purchased from Coulter. Anti-Cip1/WAF-1/p21 was purchased from Upstate Biotechnology (Lake Placid, NY); anti-phospho-Crkl (Tyr207) was from Cell Signaling Technology (Beverly, MA); anti-Crkl, anti-p27 and anti-p57 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-rabbit Ig, horseradish peroxidase (HRP)-linked whole antibody (anti-rabbit HRP-conjugated antibody) was from Amersham Pharmacia; anti-mouse HRP-conjugated antibody was from Nacalai Tesque (Kyoto, Japan).

Isolation and identification of smenospongine

Smenospongine was isolated from the marine sponge *Dactylospongia elegans* (collected in Indonesia in 2001) based on the guidance of the bioassay for induction of hemoglobin production. The dried marine sponge was cut into pieces and soaked in methanol overnight. The resulting methanol extract was subjected to solvent partition to give portions soluble in hexane, 90% methanol, *n*-BuOH and H_2O. The active 90% methanol-soluble portion was separated by repeated SiO_2 column, reversed-phase column and high-performance liquid chromatography (HPLC) to obtain an active compound. The active compound was identified as smenospongine by comparison of the mass and NMR data [11]. Smenospongine was first isolated from a marine sponge of *Smenospongia* sp. as a cytotoxic and antimicrobial sesquiterpene aminoquinone (Fig. 1) [11].

Fig. 1



Chemical structure of smenospongine.

Induction of hemoglobin production in K562 cells

To evaluate the induction of differentiation of K562 cells into erythroblasts, the pseudo-peroxidase activity of hemoglobin was measured by colorimetric assay using diaminofluorene as described in the previous report [7] except for a slight modification. The suspension of K562 cells (4×10^4 cells/2 ml/well) was placed in a 24-well plate. After addition of the test sample, the culture solution was incubated for the indicated times at 37°C under a 5% CO_2 atmosphere. Then, the cells were washed with D-PBS and crushed using 50 μl of ultra-pure water, and the diaminofluorene solution (100 μl) [5 mg/ml in a solution of 90% acetic acid and Tris–HCl buffer (pH 7.5) (10:1)] and 3% hydrogen peroxide (10 μl) were added, and kept for 30 min in the dark. The absorbance at 630 nm was measured by a microplate reader.

Flow cytometric analysis of glycophorin A expression

The cell suspension of K562 cells (2×10^5 cells/2 ml/well) was placed in an eight-well plate, and the DMSO solution (10 μl) of the test sample was added and cultured for 6 days under a 5% CO_2 atmosphere at 37°C. After harvesting the cells, 5×10^5 cells were suspended in 100 μl of D-PBS(–) solution, and the FITC-labeled glycophorin A antibody solution (20 μl) was added and further incubated for 40 min at 4°C in the dark. Then, the supernatant was removed by centrifugation (500 *g* for 5 min), and the collected cells were washed with D-PBS(–) solution and filtered with a 40-μm nylon mesh filter. The filtrate was measured by flow cytometry ($\lambda_{ex} = 494$ nm, $\lambda_{em} = 520$ nm). As for the control, the cells were treated with FITC-labeled glycophorin A antibody without the test sample.

Flow cytometric analysis of the cell cycle

The cell suspension (2×10^5 cells/2 ml/well) of K562 cells was placed in an eight-well plate and incubated for 24 h at 37°C under a 5% CO_2 atmosphere. The DMSO solution of the test sample (10 μl) was added and further incubated for the indicated time. After harvesting the cells, the collected cells were dyed using a DNA-Prep Reagents Kit for 20 min. Then, the supernatant was removed by centrifugation and the cell suspension in 500 μl of D-PBS(–) solution was filtered with a 40-μm nylon mesh filter. The cell cycle analysis of the filtrate was carried out by flow cytometry ($\lambda_{ex} = 493$ nm, $\lambda_{em} = 630$ nm) and quantified by ModFit software (Verity Software, Topsham, ME).

Western blotting analysis

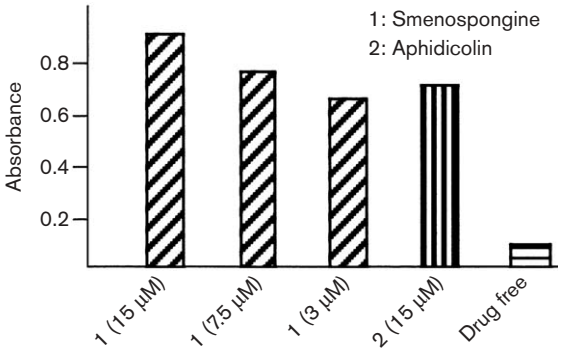
The cell suspension (1×10^6 cells/10 ml) of K562 cells was incubated in a flask together with 15 μM of smenospongine for the indicated times under 5% CO_2 at 37°C. The cells were harvested and collected by centrifugation (1000 *g* for 3 min at 4°C), washed with cold PBS and treated with lysis buffer (50 mM Tris–HCl, pH 7.2, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% proteinase inhibitor cocktail) to furnish a cell lysate. Protein assay was carried out using a Bio-Rad (Hercules, CA) protein assay kit. After boiling at 95°C for 5 min in the sample buffer (0.125 M Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 5% bromophenol blue), equal amounts of protein were subjected to SDS–PAGE and then transferred to PVDF membranes. The membrane was blocked with 5% milk TBS (Tween PBS), and exposed to the primary antibodies and then the corresponding secondary antibodies (anti-mouse or anti-rabbit IgG HRP-conjugated antibody). The bound antibodies were then visualized using an ECL system and quantified by NIH Image (NIH, Bethesda, MD).

Results

Effect of smenospongine on induction of hemoglobin production in K562 cells

The inducing activity of smenospongine for hemoglobin production in K562 cells was examined 4 days after treatment. As shown in Fig. 2, peroxidase activity, which indicates hemoglobin production in the cells, increased in response to the concentration of smenospongine (3–15 μM) or aphidicolin (15 μM) [12], respectively. Smenospongine did not show any cytotoxic effect against K562 cells at these concentrations.

Fig. 2



Induction of hemoglobin production by smenospongine. A suspension of K562 cells (4×10^4) was treated with or without test sample and cultured for 4 days. The collected cells were lysed, and then treated with diaminofluorene (5 mg/ml) and 3% hydrogen peroxide. After 30 min, the absorbance at 630 nm was measured.

Expression of glycophorin A by smenospongine treatment

The change in the expression level of glycophorin A by smenospongine treatment was analyzed in order to clarify the differentiation of K562 cells into erythroblasts by smenospongine. Glycophorin A is a major sialoglycoprotein, which is specifically expressed on the surface of human erythrocytes and erythroblasts, and is used as a marker of erythroid differentiation [13]. To measure the expression level of glycophorin A, the cells pre-treated with the drug were incubated with FITC-labeled glycophorin A antibody and the induced fluorescence was analyzed by flow cytometry. As a result, the expression of glycophorin A was significantly increased in K562 cells after 6 days in the presence of smenospongine (15 μM) or aphidicolin (15 μM) (Fig. 3). The result indicates that K562 cells treated with smenospongine differentiated cytophysiologically into erythroblasts.

Effect of smenospongine on the cell cycle in K562 cells

Since cellular differentiation and the cell cycle are closely correlated to each other, we analyzed the effect of smenospongine on cell-cycle progression. A 7.5 μM concentration of smenospongine was added to randomly cultured K562 cells and the cell cycle was analyzed after 24 h. As a result, it was found that smenospongine arrested the cell cycle of K562 cells at the G_1 phase (Fig. 4). To investigate the relation between G_1 arrest of the cell cycle and erythroid differentiation induced by smenospongine, K562 cells were incubated in the presence of smenospongine (7.5 μM) for 0, 24, 48, 72 and 96 h. The cells were collected at the indicated time points for cell-cycle analysis or hemoglobin production assay as described above. Hemoglobin production increased gradually behind the schedule of the G_1 arrest of the cell cycle (Fig. 5).

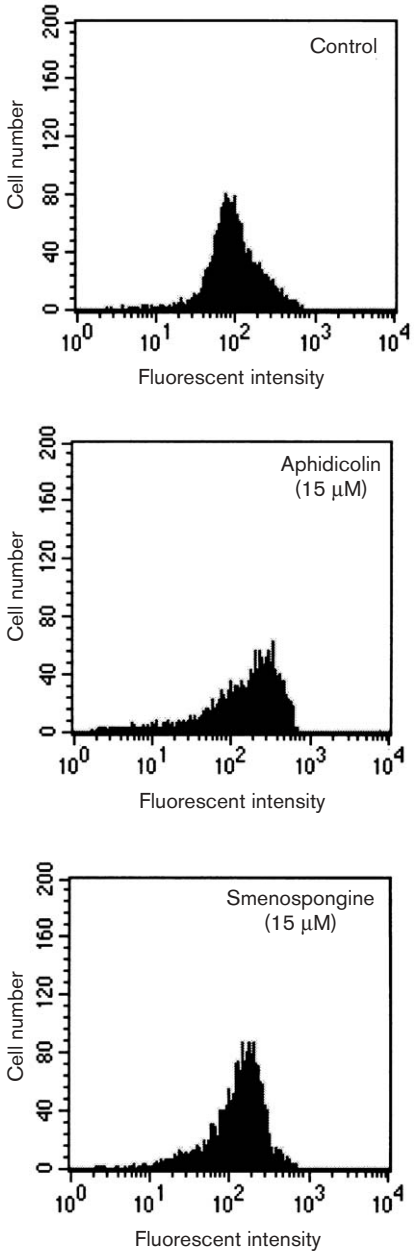
Effect of smenospongine on the expression of CIP/KIP family cyclin-dependent kinase (CDK) inhibitors (CKIs)

The effect of smenospongine on the expression of the CIP/KIP family (p21, p27 and p57) CKIs was examined [14]. A 15 μM concentration of smenospongine was added to K562 cells, and the expression of CKIs was analyzed by Western blotting and quantified by NIH Image. The expression of p21 increased after 12 h in a time-dependent manner (7-fold for 12 h, 51-fold for 24 h and 93-fold for 48 h of that for 0 h, respectively). Conversely, the expression of p57 decreased in a time-dependent manner (90% for 12 h, 58% for 24 h and 21% for 48 h of that for 0 h, respectively). There was no significant change in p27 expression (Fig. 6).

Inhibitory effect of smenospongine on phosphorylation of Crkl

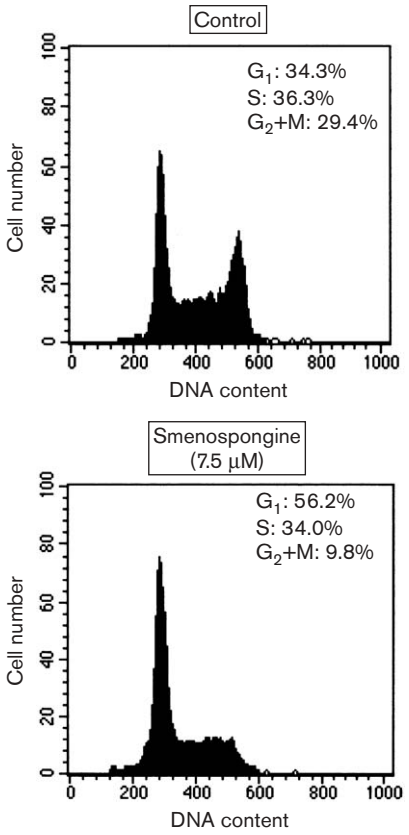
In order to examine the effect of smenospongine on Bcr–Abl tyrosine kinase, the causative protein in CML pathogenesis, we analyzed the effect of smenospongine on the phosphorylation of Crkl, which is known to be a

Fig. 3



Expression of glycophorin A in K562 cells treated by smenospongine. A suspension of K562 cells (2×10^5) was incubated with or without test sample under 5% CO_2 at 37°C. The collected cells were treated with FITC-labeled glycophorin A antibody, further incubated for 40 min in the dark and then analyzed by a flow cytometer ($\lambda_{ex}=494$ nm, $\lambda_m=520$ nm). Untreated cells were used as controls.

Fig. 4



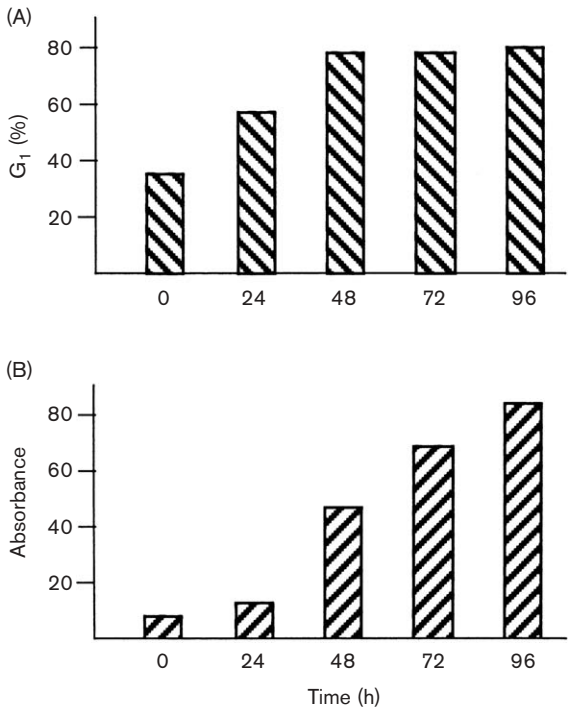
Effect of smenospongine on the cell cycle of K562 cells. A suspension of K562 cells (2×10^5) was incubated in the presence or absence of test sample under 5% CO_2 at 37°C, and, 24 h later, the cells were collected and dyed with the DNA-Prep Reagents Kit, and then analyzed by a flow cytometer ($\lambda_{ex}=493$ nm, $\lambda_{em}=630$ nm).

substrate of Bcr–Abl. Western blot analysis using the antibody against phosphorylated Crkl revealed that the phosphorylated Crkl remarkably decreased at 24 h (19% of that for 0 h) after smenospongine treatment (15 μM), indicating that the phosphorylation of Crkl by Bcr–Abl was inhibited by smenospongine, while the expression level of Crkl was not remarkably affected (92% of that for 0 h) by smenospongine (Fig. 7).

Discussion

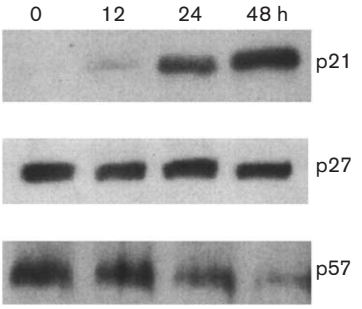
Pluripotent hematopoietic stem cells are regulated by various hormones and cytokines, and then give rise to differentiation to specialized hematopoietic cells such as erythrocytes, granulocytes, monocytes and macrophages. Leukemic cells, which arise from an abnormality in the differentiation process, cause prematurity and high proliferation. Therefore, selective destruction of cancer cells might be achieved by differentiation-inducing agents which work on this underlying abnormality of premature cancer cells [15]. The development of differentiation-induction agents arose from the observation that cultured mouse myelogenic leukemia cells differentiated into macrophage- and granulocyte-like cells by change in the culture conditions [2], and much attention has been paid to differentiation-induction therapy of cancer since the discovery of ATRA,

Fig. 5



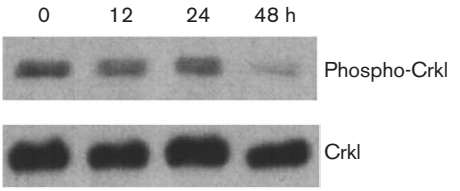
Comparison of the time course between G_1 arrest and erythroid differentiation in response to smenospongine treatment. (A) K562 cells (2×10^5) were grown in the presence of smenospongine (7.5 μM) and collected at the indicated time. Cell-cycle analysis was performed as described in Fig. 4. The percentages of the cells in G_1 phase are shown. (B) K562 cells (4×10^4) were grown in the presence of smenospongine (7.5 μM) and collected at the indicated time. Hemoglobin production was analyzed by the colorimetric method with diaminofluorene as described in Fig. 2. The absorbance at 630 nm is indicated.

Fig. 6



Effect of smenospongine on expression of CIP/KIP family CKIs. K562 cells (1×10^6) were incubated with a 15 μM concentration of smenospongine for the indicated times. Cell lysates was prepared and applied to 15–25% (for p21 and p27) or 4–20% (for p57) SDS–PAGE. After being transferred to PVDF membranes, the blots were exposed to the corresponding primary antibody and then to anti-mouse (for p21) or anti-rabbit (for p27 and p57) HRP-conjugated IgG antibody.

Fig. 7



Inhibition of phosphorylation of Crkl by smenospongine. K562 cells (1×10^6) were treated with a 15 μM concentration of smenospongine for the indicated times. Cell lysate was prepared and applied to 15–25% SDS–PAGE. After being transferred to the PVDF membrane, the blots were exposed to anti-Crkl or anti-phospho-Crkl and then to anti-rabbit HRP-conjugated IgG antibody, respectively. Equal portions of each cell lysate were analyzed by immunoblotting with anti-Crkl or anti-phospho-Crkl to confirm equal amounts of protein.

which has shown significant efficacy for the treatment of APL [3].

Differentiation-induction activity of smenospongine, a spongean sesquiterpene aminoquinone, against K562 CML cells was examined. Smenospongine showed remarkable hemoglobin production in a dose-dependent manner. The activity was stronger than that of aphidicolin [12], which is known to differentiate K562 cells into erythroblasts (Fig. 2). In order to confirm the differentiation of K562 cells by smenospongine, the time-dependent expression of glycophorin A was examined. Glycophorin A is a sialoglycoprotein specifically expressed on the cell surface in the differentiation stage of erythroblast progenitor cells and is a useful marker of erythroids [13]. The K562 cells treated with smenospongine or aphidicolin showed a significant increase of glycophorin A expression after 6 days (Fig. 3) [16,17], The remarkable increase of hemoglobin production and glycophorin A expression indicated that K562 cells treated with smenospongine differentiated into erythroblast progenitor cells.

Next, the effect of smenospongine on the cell cycle of K562 cells was examined. As shown in Fig. 4, smenospongine was found to arrest the cell cycle at the G_1 phase after 24 h. G_1 arrest along with hypophosphorylation of Rb is observed during cell differentiation in various cells [18], and this observation indicates that the arrest of the cell cycle at the G_1 phase and differentiation induction might be closely correlated. To investigate the relationship between G_1 arrest and erythroid differentiation of K562 cells induced by smenospongine, the time course of the effect on the cell cycle and hemoglobin production by smenospongine were analyzed. As shown in Fig. 5, hemoglobin production increased gradually behind the schedule of the increase of the cells arrested in the G_1

phase. This result implies that G_1 arrest of the cell cycle might play an important role in differentiation of K562 cells induced by smenospongine. Furthermore, most cancer cells exhibit abnormalities in p53 and Rb, which regulate the transition from the G_1 to S phase [19,20]. Therefore, smenospongine, which arrests the cell cycle of K562 cells at the G_1 phase, is expected to be an effective differentiation inducer, which works through discriminating cancer cells from normal cells.

In general, progression of the cell cycle is mediated by CDK–cyclin complexes and inhibited by CKIs, which suppress kinase activity of CDK–cyclin complexes. CKIs are divided into the INK4 family (p15, p16, p18 and p19) and the CIP/KIP family (p21, p27 and p57) [14] based on the primary structure and the specificity against the target CDK. The INK4 family specifically inhibits CDK4– or CDK6–cyclin D complexes and arrests the cell cycle at the G_1 phase in a Rb-dependent manner [21]. On the other hand, the CIP/KIP family has broader specificity for CDK–cyclin complexes and also arrests the cell cycle at the G_1 phase [22]. It is suggested that the CIP/KIP family may be involved in differentiation induction because of the observation of its increased expression at the early stages of differentiation [23,24]. We examined the expression of the CIP/KIP family in K562 cells treated with smenospongine. Accordingly, significant elevation of the expression of p21 was observed after 12 h, while the expression level of p57 decreased after 24 h (Fig. 6). There was no change in the expression level of p27. Trichostatin A, a histone deacetylase inhibitor, is known to induce differentiation of various cells, and to arrest the cell cycle at the G_1 and G_2 phases [25]. When trichostatin A was added to Hep 3B cells, an increase of the expression level of p21 and a decrease of p57 was observed [26]. One mechanism of action of smenospongine, which induces differentiation in K562 cells, may be achieved through increased p21 expression and G_1 arrest of the cell cycle.

Ph1, which arises from the reciprocal translocation between chromosome 9 and 22, has been found in more than 90% of CML and Bcr–Abl tyrosine kinase encoded by the resulting fusion gene is considered to cause uncontrollable proliferation in the leukemic cells [5]. The 39-kDa Crkl is a substrate of Bcr–Abl and phosphorylation of Crkl appears to initiate the signal transduction for cancerous proliferation [27]. Recently, imatinib mesylate (Glivec, ST1571, CGP57148) has been shown to be clinically effective for the therapy of CML. Thus, the inhibitory effect of smenospongine against Bcr–Abl was examined and we found that smenospongine inhibited phosphorylation of Crkl (Fig. 7). In order to assess the relationship between the inhibition of Bcr–Abl and the differentiation induction against K562 cells into erythroblasts, we also analyzed the effect of other differentiation-induction agents (aphidicolin and PHC-1), which induce differentiation against K562 cells into erythroblasts. Aphidicolin and PHC-1 did not inhibit the phosphorylation of Crkl by Bcr–Abl (data not shown). This result suggests that there is little correlation between the inhibition of Bcr–Abl and the differentiation induction into erythroblasts.

On the basis of the above evidence, smenospongine appears to exhibit two major functions: (i) to arrest the cell cycle at the G_1 phase in K562 CML cells and induce differentiation into erythroblasts, and (ii) to inhibit the phosphorylation of Crkl by Bcr–Abl. Smenospongine is expected to be a promising candidate for the treatment of CML.

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