The Root Bark of *Paeonia moutan* is a Potential Anticancer Agent in Human Oral Squamous Cell Carcinoma Cells

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Abstract. *Currently there is growing use of complementary and alternative anticancer medicines worldwide, and considerable interest in finding anticancer drugs among Chinese medicinal herbs. The aim of this study was to determine the antitumor activity of the root bark of Paeonia moutan (RBPM) in human squamous cell carcinoma (OSCC) cells. Cell lines derived from human oral squamous cell carcinoma (HSC2, 3, 4, SAS) were tested with different concentrations of RBPM (1-100 μg/ml) using a series of in vitro assay systems. RBPM at a concentration of 100 μg/ml inhibited monolayer and anchorage-independent growth, and interrupted coordinated migration. RBPM activated the phosphorylation of extracellular signal-regulated kinase (ERK) and serine/threonine kinase AKT in 30 min; then, at a later stage (after 6 hours) exhibited potent cytostatic, pro-apoptotic effects through the down-regulation of the expression of cyclin-dependent kinase 4 and its partner cyclin D1, and poly(ADP-ribose) polymerase cleavage. We found direct evidence that RBPM induces apoptotic cell death via DNA fragmentation. Taken together, the antitumor activity of RBPM was demonstrated through antiproliferative and apoptotic effects.*

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is an important part of the global burden of cancer. Although its incidence is approximately 3% of all cancers, the relative survival rates are among the lowest among major types of cancers due to a very high recurrence rate (1). The conventional treatment is a combination of surgery, radiation therapy and chemotherapy. Among these strategies against OSCC, chemotherapy with current chemotherapeutic drugs such as cis-diamminedichloroplatinum (CDDP) and 5-fluorouracil is beneficial for local control and survival improvement. However, the high incidence of severe side-effects of these drugs limits their therapeutic results (2). Thus, there has been a growing interest in developing less toxic treatments for OSCC, including complementary and alternative therapies, by identifying natural products and their active components, exhibiting chemo-preventive and chemotherapeutic potential.

Chinese medicinal herbs are considered potential sources for novel anticancer drugs (3). Discovering their active components is believed to be a promising strategy to develop more effective chemotherapies for all types of cancers, including OSCC. In our department of Showa University, an institutional collaborative project aiming at the discovery of new chemopreventive and chemotherapeutic agents from natural products, was founded in 2010 with the Tokyo University of Marine Science and Technology, from which more than 400 bioactive herbal products were provided. After screening of these herbal products by growth-inhibition assays, we focused on one Chinese medicinal herb, the root bark of *Paeonia moutan* (RBPM) of the tree peony, which exhibited potential antitumor activity. A water/ethanol extract of this root contains components shown to have various biological and biomodulating activities (4-10). However, which compounds mainly account for the pharmacological effects observed under RBPM treatment and the precise mechanisms of the antitumor effects of these compounds are still not fully understood. The purpose of this study was to elucidate the antitumor effects of RBPM on OSCC cell lines.

Materials and Methods

Preparation of the root bark of P. moutan and reagents. RBPM was imported from P.R.China. A specimen was deposited in the herbarium of the Tokyo University of Marine Science and Technology. Dry powdered roots (100 g) of RBPM were extracted

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and were then concentrated at 1 mg/ml under reduced pressure to dryness. CDDP (brand name, Randa®) was purchased from Nippon Kayaku (Tokyo, Japan). CDDP was used as positive control in each *in vitro* assay system (proliferation, migration, DNA fragmentation, and terminal deoxyuridine nick-end labeling assay) since it is the most promising antitumor (antiproliferative, apoptosis-inducing) drug for the treatment of OSCC.

Cell culture. OSCC cell lines (HSC2,3,4, SAS) derived from a human oral squamous cell carcinoma were cultured at 37˚C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Proliferation assay. Monolayer and anchorage-independent growth assays were performed as recently described (11). The monolayer cell proliferation was measured using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche Diagnostics Corp., Indianapolis, IN, USA) that measures a purple formazan compound produced by viable cells. Anchorage-independent growth was measured using a commercial kit, CytoSelect™ 96-Well *In Vitro* Tumor Sensitivity Assay (Cell BioLabs, San Diego, CA, USA), according to the manufacturer's protocol.

Migration assay. SAS cells were plated in six-well culture dishes and were grown to confluence. The tip of a 200-μl plastic pipette was used to wound the monolayer. The cells were then treated with different concentrations of RBPM (1, 10, and 100 μg/ml) and were then further cultured for 24 h. CDDP was added at a concentration of 12.5 μg/ml to block proliferation and exclude any non-migration contributions such as increased cell number. Images were captured on an inverted microscope with phase contrast.

Western blot analysis. Western blot analysis of fraction markers was carried out according to the method described previously (11). Anti-phospho-p42/p44 mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase (ERK) 1/2] and anti-phospho-p38 were obtained from Promega (Madison, WI, USA), and anti-poly(ADP-ribose) polymerase (PARP), anti-p42/p44 MAPK (ERK1/2), anti-phospho-AKT, anti-AKT, anti-phospho-c-Jun NH_2-terminal kinase (JNK), and anti-JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p38 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), from Calbiochem (Bad Soden, Germany), were purchased through BD (New Jersey, USA). Cyclin-dependent kinase (CDK) 4, cyclin D1, cell-division-cycle (CDC) 2, anti-phospho-BCL-2, anti-BCL-2 antibodies and peroxide-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

DNA fragmentation electrophoresis analysis. Genomic DNA was extracted from the cell lines using the Wizard genomic DNA purification kit (Promega), according to the manufacturer's protocol. Approximately 50 mg of DNA were loaded into each well, and 3% agarose gel electrophoresis was carried out at 100 V in a Trisacetate-EDTA (TAE) buffer for 30 min. After electrophoresis, the DNA was visualized by soaking the gel in TAE buffer containing 0.1 mg/ml ethidium bromide. The gel was observed using UV light and was photographed.

Terminal deoxyuridine nick-end labeling (TUNEL) assay. A TUNEL assay was carried out as described previously (11). Cells (5×10^4) were seeded onto Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY, USA) and incubated for 24 h. Cells were treated with RBPM (1, 10, 100 μg/ml) for 24 h; then the slides were observed using a fluorescence microscope (ECLIPSE TS100/TS100-F; Nikon, Tokyo, Japan).

Statistical analysis. Unless otherwise specified, all experiments were repeated at least twice, and similar results were obtained in the repeated experiments. Statistical analysis was carried out using the Student's *t*-test. Data are expressed as means±standard deviations, and differences were considered significant at $*p<0.05$.

Results

In vitro screening of RBPM using the monolayer and anchorage-independent growth assay in OSCC cells. According to the initial screening analyses using MTT assays, four herbal products (h201, h207, h306 and h325) were identified as candidates from more than 400 bioactive herbal products. In all established OSCC cell lines tested, the selected herb products exhibited significant growth inhibition at a dose of 100 μg/ml (Figure 1a). To further examine the growth-inhibitory activity of the four herbal products, we performed anchorage-independent growth assays using different concentrations of each selected herbal product (1-100 μg/ml). As shown in Figure 1b, two extracts, h201 and h207, were able to markedly inhibit anchorage-independent growth, at the same dose as in the monolayer growth assay. We focused on one of them, h207, and then found that the active crude compounds of interest were derived from the root bark of *P. moutan* (RBPM).

Inhibitory effect of RBPM on the motility of SAS cells. We used migration assays to test the motility of SAS cells under RBPM. Following RBPM treatment, the motility of SAS cells was significantly inhibited in a dose-dependent manner; consequently the cells, treated at a dose of 100 μg/ml, were completely unable to migrate into the wound (Figure 2).

Effect of RBPM on the activation of three subgroups of MAPK pathways and apoptosis-related signal transduction. To understand the molecular mechanism by which RBPM inhibits cell proliferation, we investigated the effects of RBPM on signal transduction. We found that RBPM at a dose of 100 μg/ml induced the phosphorylation of ERK in 30 minutes (Figure 3a), but not p38 and JNK1/JNK2. The phosphorylation of ERK persisted at 60 minutes; at 120 minutes the phosphorylation of ERK was observed to have decreased (Figure 3b). RBPM also induced the phosphorylation of AKT to a similar extent and duration, when compared to phosphorylated ERK (Figure 3b).

RBPM induces apoptotic cell death. To further investigate the molecular mechanism underlying the observed growth-suppressing effects of RBPM, we conducted assays to detect the induction of apoptotic cell death in SAS cells. As shown in

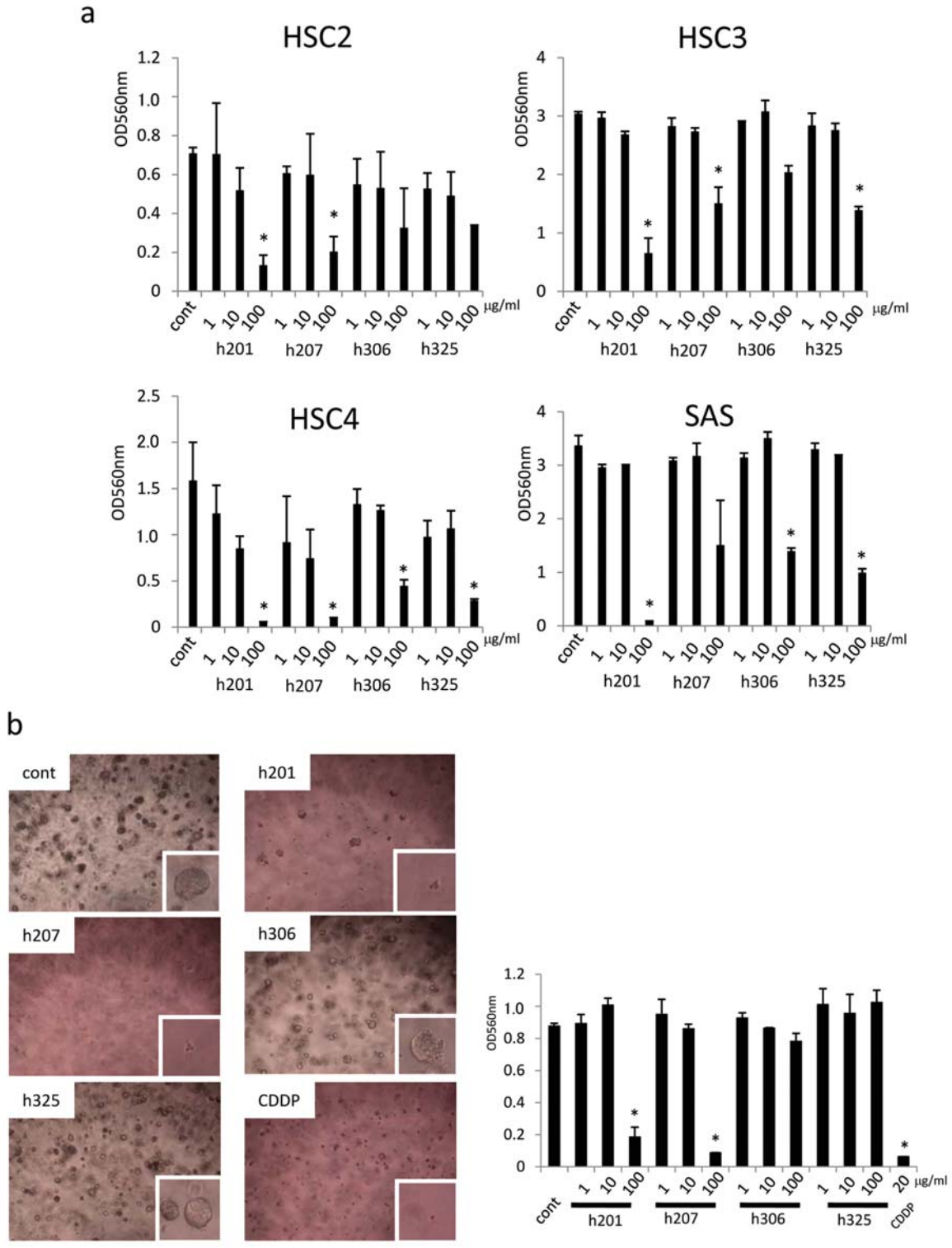

Figure 1. *Effect of the root bark of Paeonia moutan (RBPM) on the growth of oral squamous cell carcinoma (OSCC) cells. a: Screening of herb products for potent antiproliferative activities towards human OSCC cell lines. At 24 h after seeding, OSCC cells were treated with different concentrations of herb products (h201, h207, h306, and h325) and were cultured for 3 days. Asterisks indicate significant differences vs. control at *p< 0.05. b: Concentration-dependent effects of herbal products on the morphology of SAS cells. Cells incubated for 5 days with or without herbal products, as described in the Materials and Methods, were examined under a microscope and photographed with a digital CDD camera at a magnification of ×40 (insert, ×100). Asterisks indicate significant differences vs. control at *p<0.05. CDDP: Cis-diamminedichloroplatinum-exposed samples were used as positive controls.*

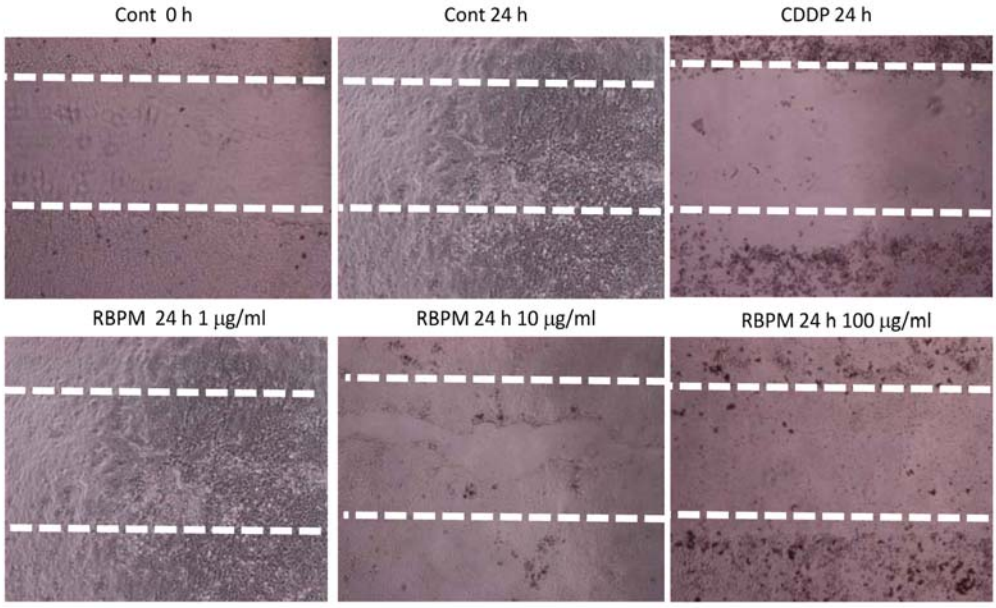

Figure 2. *Inhibitory effects of the root bark of Paeonia moutan (RBPM) on SAS cell migration. Confluent monolayers of SAS cells were wounded using a plastic pipette tip. The cells were treated with different concentrations of RBPM, then photographed after 24 h. The dotted lines highlight the margin of the scratched areas. Cont 0 h and 24 h: Samples not exposed at 0 and 24 h. Cis-diamminedichloroplatinum (CDDP)-exposed samples were used as positive controls.*

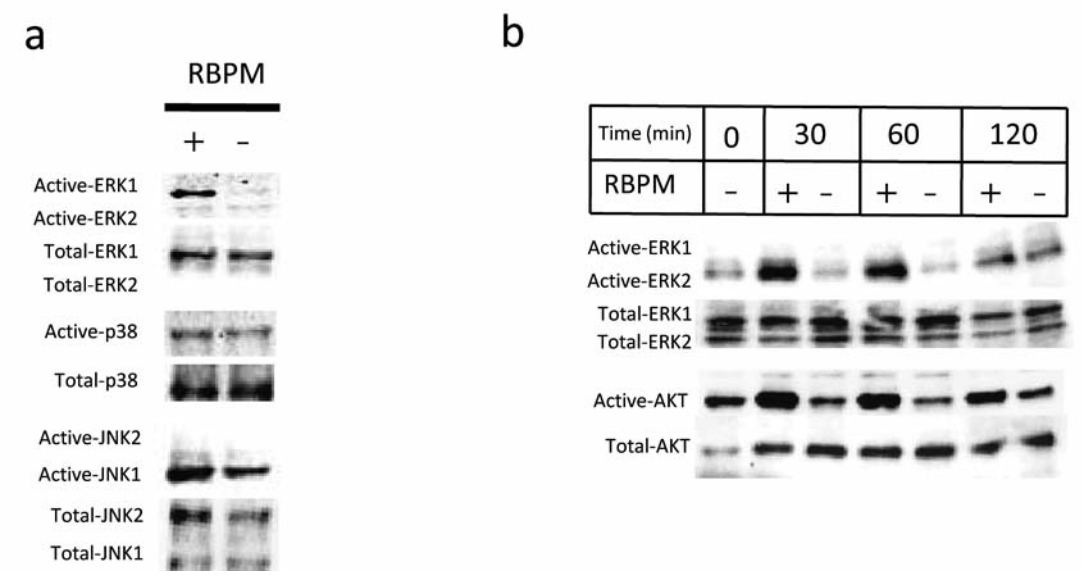

Figure 3. *Signal transduction profile in the root bark of Paeonia moutan (RBPM)-treated SAS cells. a: SAS cells were cultured after stimulation by RBPM (100 μg/ml) for 30 minutes, after which phosphorylated and total extracellular signal-regulated kinase (ERK)1/2, phosphorylated and total p38, and phosphorylated and total c-Jun NH2-terminal kinase 1/2 (JNK1/JNK2) were analyzed by western blot analysis. b: SAS cells were cultured after stimulation by RBPM (100 μg/ml) for the indicated times, after which phosphorylated and total ERK1/2, and phosphorylated and total AKT were analyzed by western blot analysis.*

Figure 4a, G_1/S-transition proteins (CDK4 and its partner cyclin D1) were depleted 6 hours after stimulation by RBPM, whereas an increase in the expression of cleaved PARP was observed at 12 and 24 h. The phosphorylation of BCL-2 also decreased at 24 h. Furthermore, DNA fragmentation was observed in SAS cells treated with RBPM at a dose of 100 μg/ml (Figure 4c). Apoptosis was directly confirmed by a significant increase in the proportion of TUNEL-positive cells (Figure 4d).

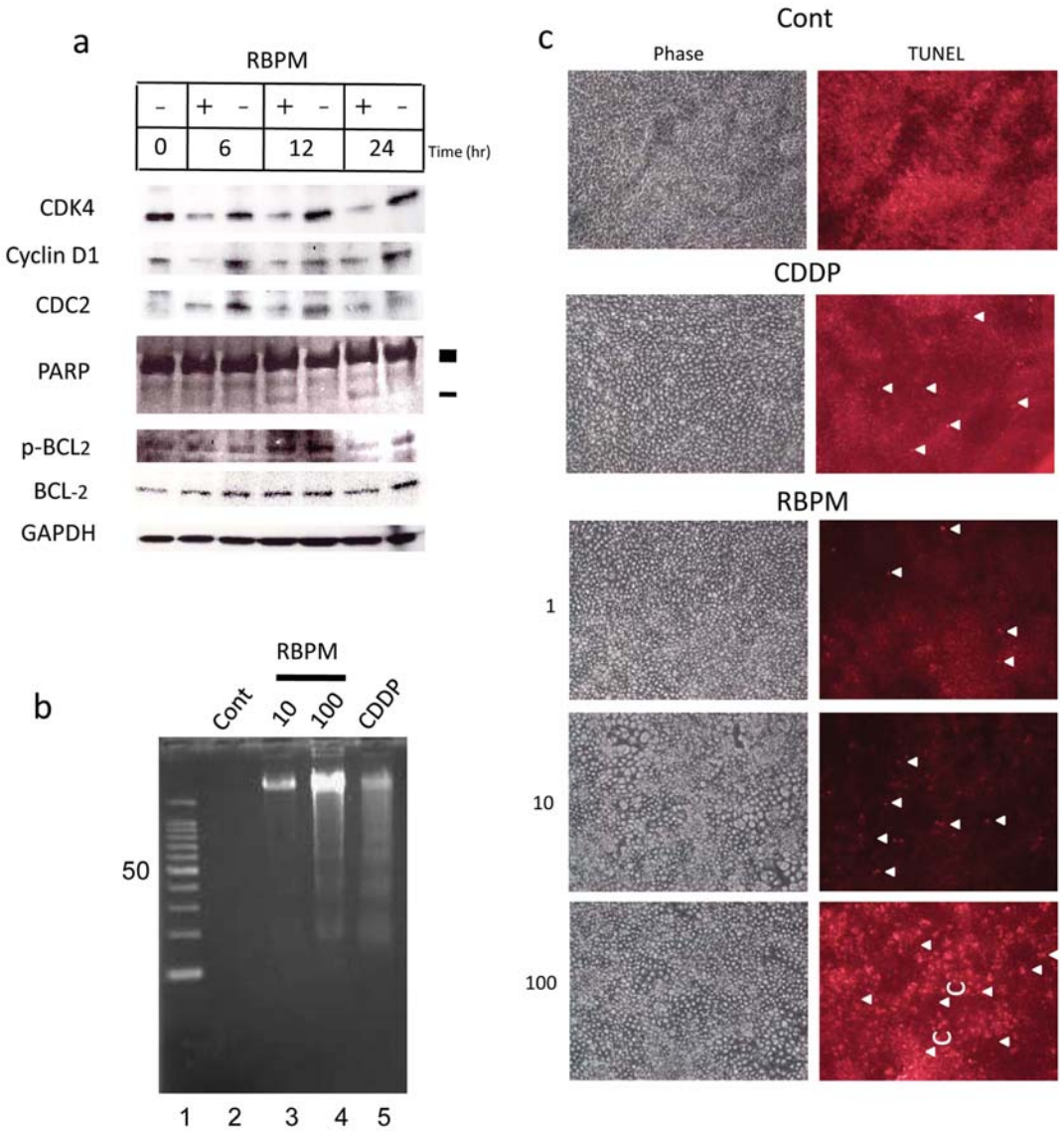

Figure 4. *Cell cycle and pro-apoptotic effect of the root bark of Paeonia moutan (RBPM) in SAS cells. a: Immunoblotting analysis of G_1/S [cyclin-dependent kinase(CDK) 4, cyclin D1], G_2/M [cell-division-cycle(CDC) 2] cell cycle transition proteins and apoptosis-related protein, BCL-2 (p-BCL-2: anti-phospho-BCL-2 antibodies, BCL-2: anti-BCL-2 antibodies), poly(ADP-ribose) polymerase (PARP) (■ full length and – cleaved fragment) expression. b: DNA fragmentation was analyzed by gel electrophoresis as described in the Materials and Methods. Lane 1, DNA marker; lane 2, normal sample; lanes 3 and 4, RBPM-exposed samples; lane 5, cis-diamminedichloroplatinum (CDDP)-exposed samples (12.5 μg/ml) as the positive control. c: Terminal deoxyuridine nick-end labeling (TUNEL) staining. SAS cells were treated with either RBPM (1, 10 or 100 μg/ml) or vehicle, for the indicated periods, and were observed under phase-contrast microscopy (Phase) and fluorescence TUNEL staining (TUNEL). White arrowheads indicate typical apoptotic cells that exhibit concentration or fragmentation of the nuclei.*

Discussion

Botanical therapeutics are included among traditional medicines. Chinese herbal preparations consist one branch of these traditional medicines. Chinese medicinal herbs have long been used to prevent and treat diseases, including cancer, and might be valid candidates for the development of antitumor drugs (3).

Peony plants, such as *P. suffruticosa*, *P. lactiflora*, *P. veitchii*, and *P. obovata*, have been used in traditional Chinese medicine and/or herbal medicine in China and Japan (4). Peony extracts contain various constituents including paeoniflorin, albiflorin, oxypaeoniflorin, benzoylpaeoniflorin, oxybenzoyl-paeoniflorin, paeoniflorigenone, lactiflorin, galloylpaeoniflorin, paeonin, paeonolide, and paeonol. Among them, paeonol, a phenolic component from the root

bark of *paeonia moutan*, is the ingredient that has so far been found, through *in vitro* and *in vivo* studies, to have the most extensive biological and biomodulating activities, including anti-inflammatory (5, 6), antiallergic (7), antiangiogenic (8) and antioxidant (9) activities. In particular, previous evidence has shown that paeonol has anti-tumor effects on human hepatocellular carcinoma cell lines (10). The concentrations of paeonol that inhibited proliferation by 50% ranged from 11.39 to 56.23 mg/l.

In the present study, for the first time, we identified a promising extract, RBPM, that has a potent antitumor activity towards human OSCC cell lines at a dose of 100 μg/ml. The relatively high bioactive concentration of RBPM required to promote such effects in our study, might be attributed to the fact that RBPM is a crude product.

To eludicate the molecular mechanism of the apoptosis provoked by RBPM, we assessed the signal transduction routes, especially those of ERK and AKT, which are both survival proteins, mutually affected throughout the sequential activation of death receptor signaling. Interestingly, RBPM was able to induce both ERK and AKT phosphorylation at an early stage (as early as 30 min, Figure 3b) which is critical for the G_1-to-S phase transition that can lead to cell proliferation. On the other hand, RBPM also induced cell cycle perturbation through its inhibition of CDK4 and cyclin D1 (Figure 4a), leading to accumulation of cells in the G_1-phase. Although the precise signaling mechanisms underlying the response to RBPM remain unclear at present, we have shown that RBPM has cytotoxic effects for OSCC cells due to the simultaneous down regulation of the phosphatidylinositol 3-kinase /AKT and RAS/ERK pathways at a late stage, although independent survival modules of the ERK and AKT pathways exist in the cell cycle. It is possible that both the ERK and AKT pathways are intracellular targets of RBPM in its cytotoxic effects on OSCC cells. Apoptosis was also induced by mitochondrial apoptotic pathways through apoptosis-associated molecules, such as BCL-2 and PARP. In fact, the cleavage of PARP and a decrease of BCL-2 expression was observed in RMPB-treated OSCC cells (Figures 4 and 5), indicating that the activation of the mitochondrial pathway during RBPM-induced apoptosis, at least in part, plays a role in RMPB-induced apoptosis. Further studies are underway to examine which of the pathways cytotoxicity in OSCC cells is more critical for the response to RBPM.

The identification of the active components of RBPM, and confirmation of their properties (*e.g.* cell cytotoxicity and apoptosis) should elucidate the precise mechanism of the antitumor effect of RBPM and may provide a basis for chemopreventive and chemotherapeutic strategies in OSCC.

Disclosure Statement

The Authors have declared that no competing financial interests exist.

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