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Differential Gene Expression in Rat Vascular Smooth Muscle Cells Following Treatment with Coptisine Exerts a Selective Antiproliferative Effect

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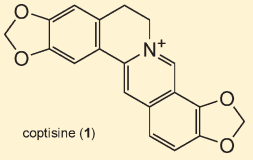
Supporting Information

ABSTRACT: It is known that coptisine (**1**), an isoquinoline alkaloid, selectively inhibits proliferation of rat primary vascular smooth muscle cells (VSMCs). In the present study, the characteristics of its antiproliferative effect on several types of smooth muscle-like cells were investigated and compared to the effects of berberine (**2**) and palmatine (**3**). To clarify further the mechanism underlying the VSMC-selective antiproliferative effect of **1**, the genes responsible were investigated by determining which mRNAs showed expression regulated by **1**. Coptisine (**1**) showed a greater antiproliferative effect on smooth muscle cells derived from the aorta than on those derived from other organs. Analysis of the mRNA expression revealed that **1** upregulated two genes, growth arrest and DNA-damage-inducible alpha (Gadd45a) and response gene to complement32 (Rgc32). Both genes remained unchanged in 3Y1 fibroblasts and were not affected by **2** and **3**. Coptisine (**1**) was found to induce the mRNA of the Gadd45a and Rgc32 genes, specifically in VSMC. Activation of these genes by **1** may mediate inhibition of cell-cycle progression. However, as these genes are commonly expressed in various cell types, a selective target for **1** activity is likely to exist upstream of these genes.



Abnormal vascular smooth muscle cell (VSMC) proliferation is considered a key event in a number of human diseases, such as coronary artery atherosclerosis, restenosis following angioplasty, and hypertension. In atherosclerosis, the change in medial VSMCs from the contractile to the synthetic state is the most widely accepted mechanism of neointimal formation to date. VSMC proliferation and differentiation are regulated intricately by a complex array of local environmental stimuli including growth factors, contractile agonists, inflammatory stimuli, and mechanical stresses.[1] Therefore, selective inhibition of the excessive proliferation of VSMCs by inducing their dedifferentiation from the synthetic to the contractile state, or by promoting apoptosis, is considered a potential preventive measure for vascular disorders. Although it is suggested that several compounds, such as tranilast,[2,3] probucol,[4] and cilostazol,[5,6] exhibit antiproliferative effects on VSMCs, there are no compounds for which the efficacy has been demonstrated by a large clinical trial. Recently, however, a drug-eluting stent was noted to prevent restenosis, and sirolimus[7] and paclitaxel[8] have proved useful in this regard. However, as drug-eluting stents also carry the risk of late stent thrombosis, concurrent long-term administration of antiplatelet drugs is required. Therefore, it is considered that the development of an antiarteriosclerosis drug targeting VSMCs is important.

To date, our group has reported that the rhizomes of *Coptis japonica* Makino (Ranunculaceae) and one of the main constituents, coptisine (**1**), selectively prevent VSMC proliferation.[9,10] However, there are no other reports about the specific interactions between **1** and VSMCs. There are a few reports detailing other aspects of **1**, for example, its gastric mucous-membrane protective activity,[11] peroxynitrite-scavenging activity,[12] and antifungal activity.[13] The other isoquinoline alkaloids contained in *C. japonica* rhizomes are berberine (**2**) and palmatine (**3**). There are some reports on the interactions between **2** and VSMCs, such as **2** suppressing MAP kinase cascade activity[14−16] and increasing AMP-activated protein kinase activity.[17] Berberine (**2**) has also been found to have effects related to diabetes, obesity, and hyperlipidemia.[18−20] In contrast, there are few reports regarding interactions between **3** and VSMC; the closest such report is that of **3** inhibiting the contractile responses stimulated by phenylephrine in rat aortic strips.[21] Regardless of the similarities in their structures, **1**−**3** exhibit different activities. Indeed, in a previous report, **2** showed lower antiproliferative activity on VSMCs than did **1** (50% growth inhibition = 95.1 μM **2**; 3.3 μM **1**), whereas **3** showed no activity.[10] These three isoquinoline alkaloids each exhibit differential effects in terms of multidrug resistance function in VSMCs.[22]

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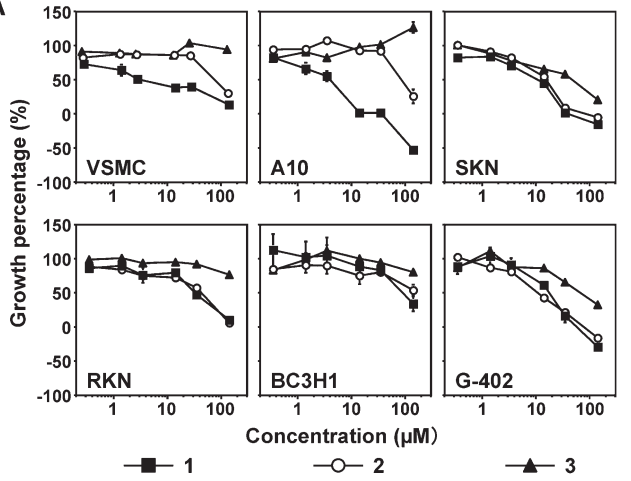
A

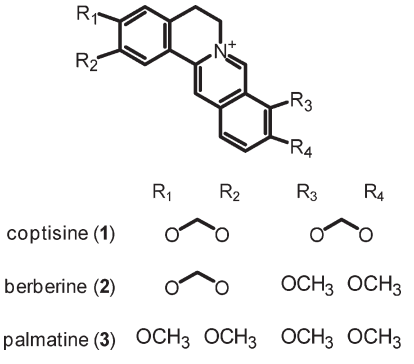


B

compound	VSMC	A10	SKN	RKN	BC3H1	G-402
1	3.35	4.49	11.76	33.02	104.94	19.10
2	95.14	101.62	15.88	49.60	—	11.87
3	—	—	57.43	—	—	83.14

(μM)

Figure 1. Effects of coptisine (**1**), berberine (**2**), and palmatine (**3**) on the proliferation of various types of smooth muscle cells. (A) Cells were seeded into 24-well plates at specified concentrations (VSMC, A10, BC3H1, and SKN, 1×10^4 cells/well; RKN and G-402, 2×10^4 cells/well) and incubated with increasing concentrations of **1**−**3**. Growth percentage was determined by MTT assay and calculated as described in the Experimental Section. Each value represents the mean ± SE of 3 wells. (B) GI_{50} values (μM) were calculated from the data in Figure 2A.



With regard to the mechanisms of the antiproliferative effect of coptisine (**1**) on VSMCs, **1** completely inhibited the induction of cyclin D1 protein in serum-stimulated VSMCs by accelerating proteasome-mediated proteolysis, which leads to cell-cycle arrest at the G_0/G_1 phase. In addition, **1** prevented progression of the cell cycle at the G_2/M phase at concentrations lower than that effective for a G_0/G_1 block.[9] Although these sites of action exist not only in VSMCs but also in other cells, the precise mechanism of action remains to be determined. In the present study, the antiproliferative effects of coptisine (**1**) on various smooth muscle-like cells were examined to elucidate its selective mechanisms. This was achieved by a global analysis of mRNA expression regulated by **1** using a VSMC A10 cell line.

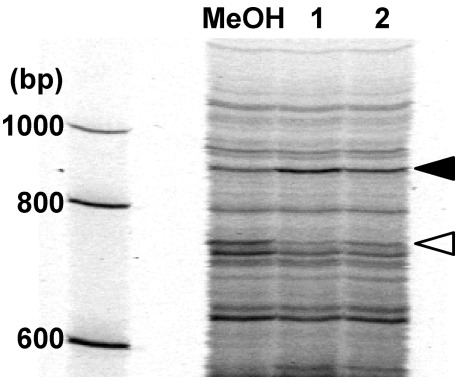


Figure 2. Example of cDNA fingerprints. A typical image of gel-separated PCR products from RNAs prepared from A10 cells treated with methanol (MeOH), 3 μM coptisine (**1**), or 30 μM berberine (**2**). In this case, arbitrary primer CMN A18 and anchor primer $G(T)_{15}A$ were used. cDNA fragments were upregulated (solid arrow) or downregulated (open arrow) following treatment with alkaloids.

RESULTS AND DISCUSSION

Effects of Isoquinoline Alkaloids on the Proliferation of Several Smooth Muscle-like Cells. It was reported previously that coptisine (**1**) exhibits an antiproliferative effect on VSMCs. The antiproliferative effects induced by **1** were investigated in several types of smooth muscle-like cells to examine the selectivity of **1** in cells derived from various tissues (Figure 1). Coptisine (**1**) showed antiproliferative effects on A10 cells (rat vascular smooth muscle cells) and rat primary vascular smooth muscle cells (VSMCs). In A10 cells and VSMCs, berberine (**2**) showed a weak antiproliferative effect and palmatine (**3**) showed no discernible effects. On the other hand, both **1** and **2** showed weak antiproliferative effects on SKN cells (human uterus leiomyoma cells), RKN cells (human ovarian leiomyosarcoma cells), and G-402 (human renal leiomyoblastoma). Using BC3H1 cells (mouse smooth muscle-like tumor cells), neither **1** nor **2** showed any antiproliferative effects. Therefore, it may be considered that the action of **1** is specific to aorta-derived VSMCs and might target a signal transduction pathway or activate a molecule specific to these VSMCs.

Global Analysis of the Alteration of mRNA Expression by Coptisine (1) and Berberine (2) in A10 Cells. To elucidate the mechanisms underlying the selective antiproliferative effect of **1** on VSMCs, the alteration of mRNA expression in A10 cells by **1** and **2** was analyzed using a fluorescent differential display. A previous study demonstrated that 3 μM (**1**) and 30 μM (**2**) block the cell-cycle progression of VSMCs at the G_2/M and G_0/G_1 phases, respectively, so **1** and **2** were evaluated at these same concentrations.[9] The PCR reactions, which were performed with 20 arbitrary primers and three fluorescent anchor primers, provided 60 PCR products for each sample. All PCR products were subjected to degenerative polyacrylamide gel electrophoresis, which revealed the same PCR products to be affected by these alkaloids (Figure 2). Coptisine (**1**) only, or both **1** and **2**, induced upregulation of 28 PCR products and downregulation of 13 PCR products (data not shown). The sequences of these affected PCR products were analyzed, and 18 known genes were identified.

Next, the mRNA expression of the 18 known genes was quantified by quantitative RT-PCR. All three isoquinoline alkaloids caused alterations in the mRNA expression of eight specific

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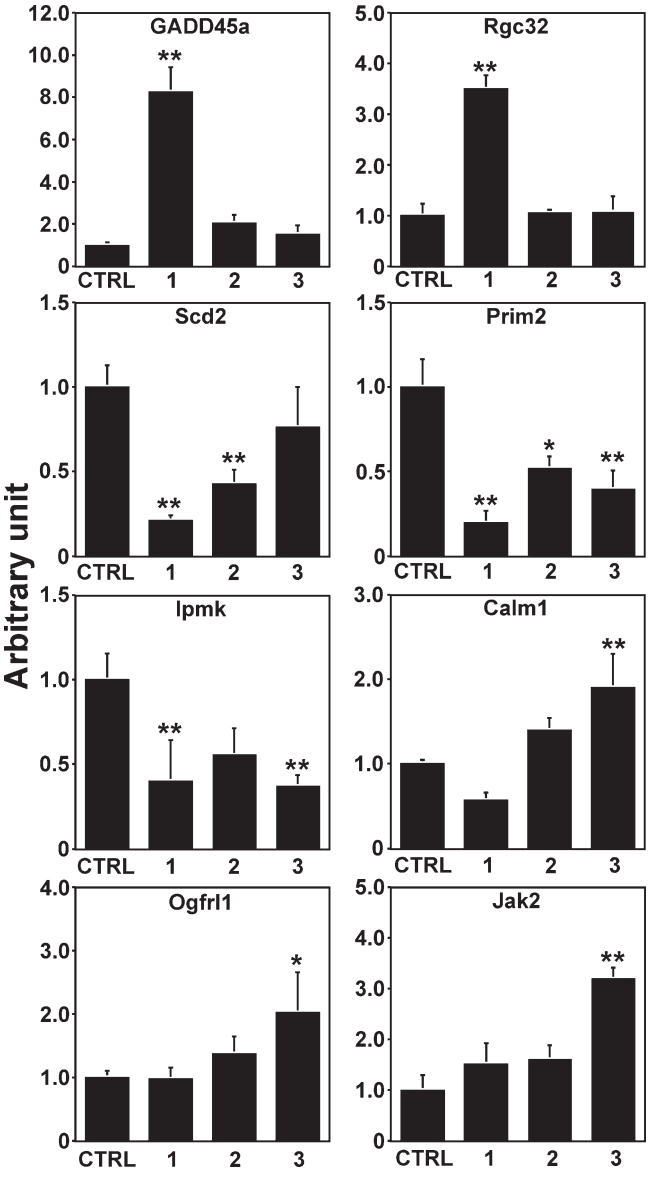

Figure 3. Effects of coptisine (**1**), berberine (**2**), and palmatine (**3**) on the expression of various mRNAs. A10 cells were seeded into 6-well plates at 6×10^4 cells/well and incubated with 30 μM of each the isoquinoline alkaloids for 24 h. Cells were harvested and mRNA levels were measured by quantitative RT-PCR. Data are expressed as the mean $\pm$ SD of 3 wells. $^*p < 0.05$, $^{**}p < 0.01$ vs the CTRL group.

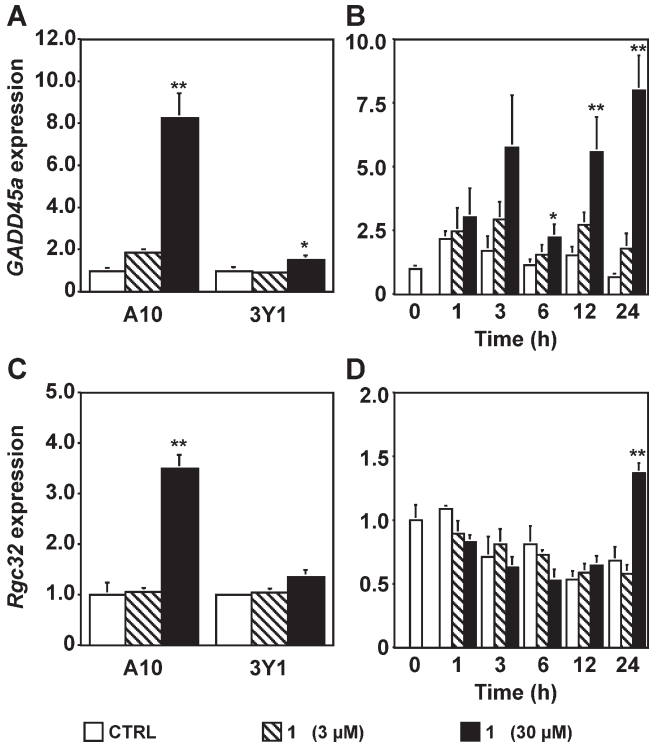

Figure 4. Effect of coptisine (**1**) on the expression on Gadd45a and Rgc32 mRNAs. (A, C) Cells were seeded into 6-well plates at specified concentrations (A10: 6.0×10^4 cells/well; 3Y1: 3.0×10^4 cells/well) and incubated with **1** for 24 h. The cells were harvested, and Gadd45a mRNA (A) and Rgc32 mRNA (C) levels were determined by quantitative RT-PCR. Data are expressed as the mean $\pm$ SD of 3 wells. $^*p < 0.05$, $^{**}p < 0.01$ vs the CTRL group. (B, D) A10 cells were seeded into 6-well plates at 6.0×10^4 cells/well and incubated with **1** for 1, 3, 6, 12, or 24 h. The cells were harvested, and Gadd45a mRNA (B) and Rgc32 mRNA (D) levels were detected by quantitative RT-PCR. Data are expressed as the mean $\pm$ SD of 3 wells. $^*p < 0.05$, $^{**}p < 0.01$ vs the CTRL group.

genes (Figure 3) as follows. Two genes found to be upregulated by **1** were identified as growth arrest and DNA-damage-inducible alpha (Gadd45a) and response gene to complement 32 (Rgc32). Three genes downregulated by all three isoquinoline alkaloids were identified as stearoyl-CoA desaturase (Scd2), primase DNA polypeptide 2 (Prim2), and inositol polyphosphate multikinase (Impk). In trun, three genes upregulated by palmatine (**3**) were identified as calmodulin 1 (Calm1), opioid growth factor receptor-like 1 (Ogfrl1), and Janus kinase 2 (Jak2).

Effects of Coptisine (1) on the Expression of Gadd45a mRNA in A10 Cells. In A10 cells, 30 μM **1** upregulated expression of Gadd45a mRNA about 8-fold compared with the control (CTRL), but a concentration of 3 μM of this compound was ineffective (Figure 4A). In 3Y1 (rat embryo fibroblast) cells used for comparison, 30 μM (**1**) showed the same effect as with the A10 cells, but its upregulation was only 1.5-fold. Next, the time-course of the Gadd45a mRNA expression was investigated in A10 cells (Figure 4B). It was found that 30 μM **1** showed a biphasic effect on Gadd45a mRNA expression, which increased for the first 3 h following the addition of **1**, then was decreased at 6 h before increasing again thereafter.

It has been reported that Gadd45a is upregulated between the S and M phases of the cell cycle in VSMCs following stimulation by serum or platelet-derived growth factor-BB.[23] Therefore, it may be considered that the expression of Gadd45a is cell cycle dependent. In the present study, the cell cycle was not synchronized, and therefore further experiments are needed to determine whether the upregulation of Gadd45a by **1** affects cell-cycle progression. Our previous study showed that **1** arrested cell-cycle progression at the G_2/M phase when this alkaloid was added to the cell population in the S phase. Cell-cycle progression between the S and M phases occurs rapidly; therefore, it is feasible that Gadd45a mRNA upregulation 1 h after adding **1** might mediate arrest of the cell cycle at the G_2/M phase.

Effects of Coptisine (1) on the Expression of Rgc32 mRNA in A10 Cells. In A10 cells, 30 μM (**1**) upregulated Rgc32 mRNA expression 3.5-fold compared with the CTRL, but 3 μM (**1**) was

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ineffective (Figure 4C). In 3Y1 cells, coptisine showed no effect on Rgc32 mRNA expression. The time-course of Rgc32 mRNA expression in A10 cells was investigated (Figure 4D). No differences were seen for the first 12 h after the addition of 30 μM **1**, with increased expression seen only at 24 h.

Rgc32 is reported to be both a positive and negative regulator of cell-cycle progression. In VSMCs and vascular endothelial cells, Rgc32 progresses the cell cycle by activating the cell division control protein 2 homologue,[24,25] but in tumor cells and vascular endothelial cells, cell-cycle progression is induced by p53 or hypoxia, respectively, and cell-cycle regulation by Rgc32 is negative.[26,27] Therefore, the particular effects exhibited by Rgc32 are determined by experimental conditions; cell-cycle progression is positively regulated by proliferative stimulation by serum, while, on the other hand, it is negatively regulated by DNA damage or in response to stress. It has not been determined previously whether the upregulation of Rgc32 by **1** positively or negatively regulates cell-cycle progression in VSMCs. In this study, berberine (**2**) and palmatine (**3**) showed no effect on Rgc32 mRNA expression; only **1** was seen to regulate Rgc32. Therefore, as **1** was also antiproliferative, upregulation of Rgc32 mRNA expression by **1** may mediate cell-cycle arrest. We therefore examined whether or not Gadd45a and/or Rgc32 are involved in the regulation of cell proliferation and cell-cycle progression by transiently overexpressing Gadd45a or Rgc32 mRNA in A10 cells. A10 cells that overexpress Gadd45a or Rgc32 mRNA exhibited a tendency to accumulate in the G2/M phase (p value = 0.07 and 0.10, respectively). These results suggest that Gadd45a and RGC32 affect the cell-cycle progression of VSMCs.

Effects of Coptisine (1) on the Expression of p53 and p21 mRNA in A10 Cells. Next, the expression of p53, which regulates both Gadd45a and Rgc32, was investigated. Coptisine (**1**) exhibited no effect on p53 mRNA expression in VSMCs, but upregulated p21 mRNA, which is tightly regulated by p53 (Figure S1, Supporting Information). It was deduced therefore that p53 activity may also participate in the **1**-induced antiproliferative effect in VSMCs. It is well known that p53 is activated in response to DNA damage, and as such, the influence of **1** on DNA or RNA may lead to the activation of p53. To date, both berberine (**2**) and palmatine (**3**) have been reported to bind to AT-rich DNA[28,29] and the poly(A) of mRNA,[30] but the same binding activity has not been reported for **1**. Some reports show that **1** binds to particular sequences of DNA with a stronger affinity than **2**,[31] and this finding suggests sequence-dependent binding of **1** and **2** to DNA. Thus, **1** may act specifically on VSMCs by binding to DNA at which neither **2** nor **3** can bind.

In the present study, mRNAs such as Gadd45a, Rgc32, and p21 that are upregulated specifically by **1** have been identified for the first time. Furthermore, coptisine (**1**)-induced mRNA upregulation is specific to VSMCs, which suggests that this mediates VSMC-specific antiproliferation. Interestingly, p53, which is the common upstream factor for Gadd45a, Rgc32, and p21, exists not only in VSMCs but also in other cells. This means that **1** may act at VSMC-specific signal transduction pathways or proteins that are upstream of p53. In other words, **1** upregulates Gadd45a, Rgc32, and p21 mRNA in VSMCs. However, as it is reported that the activities of Gadd45a, Rgc32, and p21 are either dependent or not dependent on p53,[32,33] **1** might upregulate p53 in an independent manner. Coptisine (**1**) demonstrated upregulation of Gadd45a, p21, and Rgc32 mRNA expression at 1, 6, and 24 h, respectively.

EXPERIMENTAL SECTION

Reagents. Fetal calf serum (FCS) was obtained from Nichirei Biosciences (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) with high glucose, minimum essential medium Eagle medium (MEM), antibiotics (penicillin and streptomycin), and G418 were obtained from Sigma-Aldrich (St. Louis, MO). Ham's F12 medium, coptisine (**1**) chloride (98%), berberine (**2**) chloride (99%) and palmatine (**3**) chloride (99%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). RNAiso Plus was from Takara (Shiga, Japan). McCoy's 5a medium, DNaseI, and Super Script II reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). Taq DNA polymerase was from Roche Diagnotics (Basel, Switzerland). Gene Taq DNA polymerase was from Nippon Gene (Tokyo, Japan). pGEM-T Easy Vector System was from Promega (Madison, WI). Arbitrary primers (CMN-A00 to A19) were from BEX Co. (Tokyo, Japan). All other reagents were analytical reagent grade purchased from commercial sources.

Cell Culture. Aortic VSMCs were isolated from the thoracic and abdominal aortas of 10-week-old male Sprague−Dawley rats using the enzymatic method described previously.[9,10] VSMCs were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. VSMCs were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The identity of VSMCs was confirmed by morphological examination and by RT-PCR analysis of *l*-caldesmon, *h*-caldesmon, and smooth muscle myosin heavy chain-1. Cells at passages between 5 and 15 were used for the present experiments.

A10 (rat vascular smooth muscle cell) and BC3H1 (mouse brain tumor; smooth muscle-like) cell lines were obtained from the American Type Culture Collection (Manassas, VA). SKN (human uterus leiomyoma), RKN (human ovarian leiomyosarcoma), G-402 (human renal leiomyoblastoma), and 3Y1 (rat embryo fibroblast) were obtained from Health Science Research Resources Bank (Osaka, Japan). A10 and BC3H1 were maintained in DMEM, SKN and RKN were maintained in Ham's F12 medium, G-402 was maintained in McCoy's 5a medium, and 3Y1 was maintained in MEM supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin. All cultures were then maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation Assay. Cells were seeded into 24-well plates at specified concentrations (VSMC, A10, BC3H1, and SKN: 1×10^4 cells/well; RKN and G-402: 2×10^4 cells/well) and cultured for 24 h. Next, coptisine (**1**), berberine (**2**), and palmatine (**3**) were added to the cultures, which were then incubated for a further 72 h (VSMC, A10, BC3H1, and SKN) or 48 h (RKN and G-402). After exchanging the medium with fresh medium, 500 μL of MTT (1 mg/mL for VSMC, A10, and BC3H1; 400 μg/mL for SKN; and 200 μg/mL for RKN and G-402) was added to each well, with the cells incubated for another 4 h. Following the solubilization of the formed formazan with sodium dodecyl sulfate, the absorbance was measured at 570 nm and the cell growth percentage (GP) was calculated according to the following formula: GP (>0%) = [(absorbance of cells treated with an alkaloid) − (absorbance at time 0)]/[(absorbance of control cells) − (absorbance at time 0)]; GP (<0%) = [(absorbance of cells treated with an alkaloid) − (absorbance at time 0)]/(absorbance at time 0).

Fluorescent Differential Display. Total RNA from A10 cells was extracted using RNAiso Plus according to the manufacturer's instructions. The extracted RNA was treated with DNaseI to degrade contaminating DNA. The RNA was dissolved in diethyl pyrocarbonate-treated water and quantified. To prepare first-strand cDNA, 2.5 μg of total RNA was denatured at 70 °C for 10 min with 5 pmol of FITC-labeled 3′-anchored oligo-dT primer; $G(T)_{15}A$, $G(T)_{15}C$, or $G(T)_{15}G$; 10 mM DTT; and 40 U RNase inhibitor, then reverse-transcribed with 200 U Super Script II reverse transcriptase and 0.5 mM dNTPs. Reverse

transcriptase reaction conditions were as follows: 25 °C for 10 min, 40 °C for 60 min, and 70 °C for 15 min. Next, cDNA prepared by the reverse transcription reaction was subjected to PCR with 0.5 μM anchored primer, 0.4 μM arbitrary primer, 200 μM of each dNTP, 0.5 U of Gene Taq DNA polymerase, and 0.02 U of Taq DNA polymerase. The PCR conditions were as follows: 94 °C for 3 min, 44 °C for 5 min, and 72 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 44 °C for 2 min, and 72 °C for 1 min, with an additional extension step at 72 °C for 5 min. PCR products were separated on a 4% polyacrylamide/7 M urea gel and analyzed by FluorImager (GE Healthcare, Amersham, Buckinghamshire, UK). The bands of interest were excised from the gel, and the cDNA was eluted with distilled water and reamplified by PCR with appropriate primer pairs. Reamplification products were cloned into a pGEM-T easy vector according to the manufacturer's instructions. The inserted cDNA was sequenced with a 3130 genetic analyzer (Applied Biosystems, CA) and identified with the BLAST program to search the GenBank database.

Quantitative RT-PCR. Total RNA from the VSMCs was extracted using RNAiso Plus reagent according to the manufacturer's instructions. The extracted RNA was treated with DNaseI to degrade contaminating DNA. The RNA was dissolved in diethyl pyrocarbonate-treated water and quantified. To prepare first-strand cDNA, 500 ng of total RNA was reverse-transcribed using ReverTra Ace (Toyobo Co., Osaka, Japan) according to the manufacturer's instructions. cDNA prepared by the reverse transcription reaction was subjected to PCR amplification in a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) with SYBR Green PCR Master Mix using specific primers. The primer sequences are depicted in Table S1, Supporting Information. The value calculated by cycle number is expressed as a ratio to the value for 18S rRNA amplified from the same aliquot as that used for the RT reaction.

Statistical Analysis. Data are presented as means ± SD. Differences between groups were analyzed for significance by ANOVA followed by Bonferroni-type multiple *t*-tests.

■ ASSOCIATED CONTENT

Supporting Information. Figure S showing the effect of coptisine (**1**) on the expression of p21 mRNAs, and Table S1 giving the sequences of the specific primers used for quantitative RT-PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

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