Selective Regulation of Multidrug Resistance Protein in Vascular Smooth Muscle Cells by the Isoquinoline Alkaloid Coptisine

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When the biological activites of hydrophobic drugs or xenobiotics are studied, it is important to clarify their effects on expression and function of multidrug resistance (MDR) protein. We therefore evaluated the effects of coptisine on MDR in comparison with the structurally related isoquinoline alkaloids berberine and palmatine. To achieve this, we investigated the effects of the three alkaloids on the expression and function of P-glycoprotein/MDR1, *MDR1* gene products, in vascular smooth muscle cells (VSMCs). In A10 cells (a rat VSMC line), coptisine upregulated the mRNAs of *Mdr1a* and *Mdr1b*, rodent homologues of human *MDR1*, and these effects were completely abrogated by actinomycin D. Coptisine also induced Mdr1a/1b protein expression and enhanced the efflux of rhodamine 123 from A10 cells. In contrast, berberine and palmatine slightly upregulated the mRNAs of *Mdr1a* and *Mdr1b*, but failed to induce Mdr1a/1b protein expression or stimulate rhodamine 123 efflux. To clarify whether these effects occurred in other cells, the effects of the three alkaloids on Mdr1a/1b function were examined in 3Y1, dRLh-84 and B16 cells. Coptisine and berberine enhanced rhodamine 123 efflux in all three cell types, while palmatine inhibited it, based on the finding that palmatine efficiently activated the Mdr1a ATPase activity as a good substrate for Mdr1a. Therefore, the three isoquinoline alkaloids regulated MDR differently in cell type-specific manners. In particular, only coptisine induced Mdr1a/1b in A10 cells and stimulated rhodamine 123 efflux. Taken together, coptisine appears to exert VSMC-selective effects on Mdr1a/1b induction in contrast to berberine and palmatine.

Key words coptisine; isoquinoline alkaloid; multidrug resistance protein; berberine; palmatine

Isoquinoline alkaloids such as coptisine, berberine and palmatine isolated from the rhizome of *Coptis japonica* Makino have been demonstrated to exert wide ranges of biological and pharmacological activities. In particular, the major medically important isoquinoline alkaloid, berberine, has been extensively studied[1—9] and recently reported to display beneficial effects in the treatment of diabetes, obesity and hyperlipidemia,[10—12] while both coptisine and palmatine have been reported to exhibit a limited number of biological activities compared with berberine.[13—18] These three isoquinoline alkaloids possess the same tetracyclic structure but differ in the nature of the substituents on the benzo ring, comprising methylene dioxy and/or dimethoxy substituents (Fig. 1). Regardless of the similarities in their structures, numerous studies have indicated that these three isoquinoline alkaloids appear to possess distinct activities with different potencies. For example, the potencies of the antibacterial activities against intestinal bacteria are in the order of berberine, palmatine and coptisine.[5] The inhibitory effect on dopamine biosynthesis in PC12 cells is observed with berberine and palmatine, but not with coptisine.[19] The inhibitory activity of acetylcholinesterase and peroxynitrite scavenging activity are seen similarly with three alkaloids.[20] Regarding the aldose reductase inhibitory activities,[12] hydroxy radical scavenging activities[7] and H^+, K^+-ATPase inhibitory activities,[8] coptisine exhibits more potent activity than berberine and palmatine. Previous our study has also demonstrated that coptisine is much more potent than berberine in suppressing proliferation of vascular smooth muscle cells (VSMCs), while palmatine failed to show any inhibitory activity.[16,21] Taken together, the biological activity of these three alkaloids is unlikely to be completely explained by only the slight difference in hydrophobicity based on their structures. Rather, the characteristic structures of each alkaloid appear to be crucial to their respective activities.

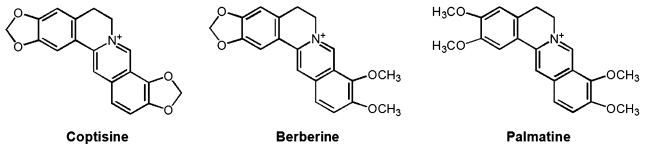

Fig. 1. Structures of Coptisine and the Related Isoquinoline Alkaloids Berberine and Palmatine

P-glycoprotein/MDR1, a 170-kDa membrane protein encoded by the *ABCB1*/*MDR1* gene, was the first ATP-binding cassette (ABC) transporter to be identified and has become the most studied gene in the field of multidrug resistance (MDR).[22] Immunohistological studies show that MDR1 highly expresses in specialized epithelial cells with secretory/excretory functions such as epithelial cells lining the gastrointestinal tract and kidney and in endothelial cells of capillary blood vessels at blood-brain and other blood-tissue barrier sites.[23,24] The expression pattern of MDR1 suggests that its major physiological role is to serve as a barrier to entry of xenobiotics including hydrophobic and amphipathic drugs into body, to remove xenobiotics from the circulation once they have entered, and to keep drugs from leaving the circulation into tissues that are especially sensitive to their toxicities.[25] Furthermore, MDR1 is highly expressed in drug-resistant cancer cells, especially cancer cells derived from cells that normally express MDR1.[26] Since MDR1 overexpression is broadly correlated with drug resistance in many different forms of cancer, effective regulation of MDR has been a long-term objective for improving the efficacy of

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chemotherapeutic agents.

Regarding the effects of isoquinoline alkaloids on MDR1 expression and function, berberine is known to not only enhance the expression and function of MDR1 on oral, gastric and colon cancer cells and hepatoma cells[27,28] but also act as an MDR1 substrate.[29] On the other hand, palmatine has been reported to inhibit MDR1 function in uterine sarcoma and colon carcinoma-derived cell lines.[30] However, it is not known that coptisine influences MDR1 expression and function in cancer cells as well as normal cells. Many studies concerning MDR focused on epithelial and endothelial cells lining the surface of organs as normal cells, but not VSMCs localized under epithelial and endothelial cells. The abnormal and excessive proliferation of VSMCs has long been considered a critical event in the pathology of coronary artery atherosclerosis, restenosis following angioplasty, and hypertension.[31] We are now attempting to treat atherosclerosis and restenosis following angioplasty with coptisine, which shows selective antiproliferative effect on VSMCs.[16,21] When hydrophobic drugs or xenobiotics with various biological and pharmacological activities are applied to various diseases, it is necessary to clarify the effect on MDR. Therefore, in the present study we examined the effects of coptisine on Mdr1a/1b, rat homologues of human MDR1, especially in VSMCs compared with those of berberine and palmatine, with the aim of elucidating the difference in biological and pharmacological activities among coptisine, berberine and palmatine.

MATERIALS AND METHODS

Chemicals and Reagents Fetal calf serum (FCS) was obtained from Nippon Bio-Supply Center (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) with high glucose and antibiotics (penicillin and streptomycin) was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). An anti-MDR (H-241) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Trizol reagent and DNaseI were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Taq DNA polymerase, protease inhibitor cocktail tablets and CDP-Star™ were purchased from Roche Diagnostics (Basel, Switzerland). Ampli Taq Gold DNA polymerase and SYBR Green PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, U.S.A.). Coptisine, berberine and palmatine were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical reagent grade and purchased from commercial sources.

Cell Culture A10 cells (a rat VSMC line) were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), and cultured in DMEM supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. B16 (mouse skin melanoma cell line), 3Y1 (rat embryo fibroblast cell line) and dRLh-84 (rat hepatoma cell line) cells were obtained from the Health Science Research Resources Bank (Osaka, Japan), and cultured in minimal essential medium (MEM)-Eagle's salt medium supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. All four cell types were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Western Blot Analysis After various treatments, VSMCs were harvested and lysed in 10 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100, 0.15 mM KCl, 5 mM 2-mercaptoethanol, 1.3 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitor cocktail tablets. The cell lysates were incubated on ice for 30 min with vortexing every 5 min, and then cleared by centrifugation at 12000×g for 15 min at 4 °C. The protein concentrations of the extracts were determined using the bicinchoninic acid (BCA) protein assay reagent. Aliquots of the extracts (25 μg protein) were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) using an 8% gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, U.S.A.). The membrane was blocked in a solution of 5% powdered skim milk in Tris-buffered saline (TBS) for 2 h and then incubated with the anti-Mdr (H-241) antibody (1 : 1000 dilution) in TBS containing 1% powdered skim milk and 0.05% Tween-20 overnight at 4 °C. The membrane was washed three times with wash buffer (0.05% Tween-20 in TBS) and then incubated with an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Cell Signaling, Beverly, MA, U.S.A.) in TBS containing 1% powdered skim milk and 0.05% Tween-20 for 1 h at room temperature. The membrane was thoroughly washed three times with wash buffer, and antibody-bound proteins were detected using CDP-Star™ as the substrate for alkaline phosphatase.

Quantitative Reverse Tanscriptase-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from VSMCs using Trizol 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. The obtained cDNA was subjected to PCR amplification using a 7300 Real-Time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix using the following specific primers: *Mdr1a* sense primer, 5′-GCT GTG GGA AAA GCA CAA CTG-3′ and antisense primer, 5′-CCG CAG ATA CCT CAC ATT GAT G-3′; *Mdr1b* sense primer, 5′-GGG AAC TCT CGC TGC TAT CAT C-3′ and antisense primer, 5′-TTT ACC CAA GCA GAG ACC CG-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* sense primer, 5′-GTG ACA AAG TGG ACA TTG TTG-3′ and antisense primer, 5′-ATG AGC CCT TCC ACG ATG C-3′. The value calculated by the cycle number was expressed as a ratio to the value for *GAPDH* amplified from an aliquot of the same RT-PCR amplification.

Measurement of Mdr1a/1b Activity Using Rhodamine 123 Cells were seeded into 12-well plates at specified concentrations (A10 cells: 3.0×10^4 cells/well; dRLh-84: 1.2×10^4 cells/well; 3Y1: 1.5×10^4 cells/well; B16: 2.5×10^4 cells/well) and cultured in DMEM supplemented with 10% FCS. Following 48 h of culture, the cells were treated with 30 μM coptisine, berberine or palmatine for 24 h. The cells were then incubated in fresh DMEM for 30 min, before the medium was replaced with DMEM containing 5 μM rhodamine 123. After 2 h of incubation, the cells were harvested and washed with ice-cold Hanks' balanced salt solution (HBSS). The intracellular concentrations of rhodamine 123

were measured by flow cytometry (FACSCant; Becton Dickinson, Mountain View, CA, U.S.A.).

Effects of Isoquinoline Alkaloids on the ATPase Activity of Rat Mdr1a MDR-dependent ATPase activity was measured using BD Gentest™ rat Mdr1a membranes according to the manufacturer's instructions with some modifications. Briefly, membranes (20 μg/assay) were preincubated in the assay buffer for 3 min. The reaction was initiated by the addition of 3 mM Mg-ATP and allowed to continue for 20 min. The reaction was stopped by the addition of 10% sodium dodecyl sulfate and the released inorganic phosphate was measured as described in the manufacturer's instructions.

Statistical Analysis The data are presented as means±S.D. The statistical significance of differences between groups was determined by ANOVA followed by a Bonferroni-type multiple *t*-test.

RESULTS

Effects of Isoquinoline Alkaloids on the Expressions of Mdr1a/1b in A10 Cells To examine the effects of coptisine on MDR, the protein levels of MDR1a/1b in A10 cells were determined by Western blot analysis (Fig. 2A). Following 24 h of treatment, coptisine increased the MDR1a/1b protein level by approximately three-fold compared with control cells, while berberine and palmatine had no effects. When the *Mdr1a* and *Mdr1b* mRNA levels were determined by real-time RT-PCR, coptisine was found to increase the mRNA levels of both *Mdr1a* and *Mdr1b* (Fig. 2B) and these effects were blocked by actinomycin D, a transcriptome inhibitor (Fig. 2C). Both berberine and palmatine slightly increased the mRNA levels of *Mdr1a* and *Mdr1b*, although neither of these alkaloids affected the Mdr1a/1b protein level. These results demonstrate that coptisine is capable of enhancing Mdr1a/1b expression in A10 cells.

Effects of Isoquinoline Alkaloids on the Function of Mdr1a/1b in A10 Cells To examine whether the Mdr1a/1b protein induced by coptisine was functional, we measured the intracellular concentration of rhodamine 123, a fluorescent substrate of Mdr1a/1b, in A10 cells after loading rhodamine 123 into the cells following treatment with coptisine. Coptisine treatment reduced the intracellular concentration of rhodamine 123 to approximately 60% of the level in control cells (Fig. 3). In contrast, berberine and palmatine had no effects on the intracellular concentration of rhodamine 123.

Effects of Isoquinoline Alkaloids on the Function of Mdr1a/1b in Various Cells Next, we assessed the effects of coptisine on Mdr1a/1b function in various cell types including dRLh-84, 3Y1 and B16 cells. As it is well known that there is a significant correlation between *Mdr1* expression and rhodamine 123 efflux in many cells, rhodamine 123 allows a nonradioacitve method for determining *Mdr1* function by flow cytometric analysis.[32] In all three kinds of cells, the three isoquinoline alkaloids increased the Mdr1a/1b protein level (data not shown), in contrast to the results for A10 cells. In addition, both coptisine and berberine significantly reduced the intracellular concentration of rhodamine 123, while palmatine increased it (Fig. 4).

Effects of Isoquinoline Alkaloids on the ATPase Activity of Rat Mdr1a Mdr1a/1b is a transporter driven by the energy of ATP hydrolysis. The substrate for Mdr1a/1b can bind to ATPase and activate its activity. Therefore, we examined the effects of the three isoquinoline alkaloids on the ATPase activity of rat Mdr1a (Fig. 5). The results revealed that verapamil, a well-known competitive substrate of Mdr1a/1b, highly activated the ATPase activity (31.01 nmol/mg/min for 20 μM verapamil), whereas coptisine slightly activated the activity (5.86 nmol/mg/min for 30 μM coptisine), thereby indicating that coptisine was a poor substrate of Mdr1a. However, both berberine and palmatine activated the ATPase activity in dose-dependent manners and appeared to serve as substrates for Mdr1a (8.42 nmol/mg/min for 30 μM berberine and 16.59 nmol/mg/min for 30 μM palmatine).

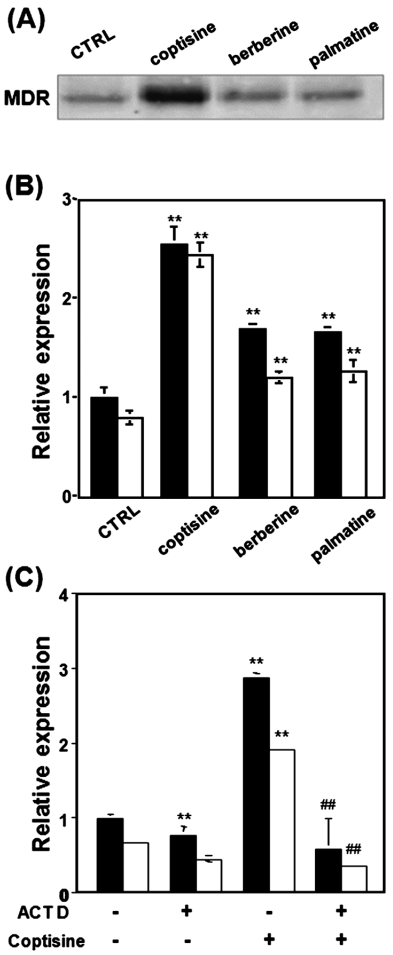

Fig. 2. Effects of Isoquinoline Alkaloids on MDR Protein and mRNA Expressions

(A) A10 cells were seeded into 10-cm dishes at 3×10^5 cells/dish, cultured for 72 h and then incubated with isoquinoline alkaloids at 30 μM for 24 h. The cells were harvested and lysed, and aliquots (25 μg protein) were subjected to SDS-PAGE. MDR1a/1b was detected by Western blot analysis. (B) A10 cells were seeded into 10-cm dishes at 3×10^5 cells/dish, cultured for 72 h and then incubated with isoquinoline alkaloids at 30 μM for 6 h. The cells were harvested and the *Mdr1a* and *Mdr1b* mRNA levels were detected by quantitative RT-PCR. Closed columns: *Mdr1a*; open columns: *Mdr1b*. Data are expressed as the means±S.D. of three wells. ** $p<0.01$ *vs.* the CTRL group. (C) A10 cells were seeded into 10-cm dishes at 3×10^5 cells/dish, and cultured for 72 h. Next, 40 μM actinomycin D (ACT D) was added to the cultures and preincubated for 10 min, followed by incubation with isoquinoline alkaloids at 30 μM for 6 h. Closed columns: *Mdr1a*; open columns: *Mdr1b.* Data are expressed as the means±S.D. of three wells. ** $p<0.01$ *vs.* the CTRL group; ## $p<0.01$ *vs.* the coptisine group.

DISCUSSION

In the present study we first attempted to evaluate the dif-

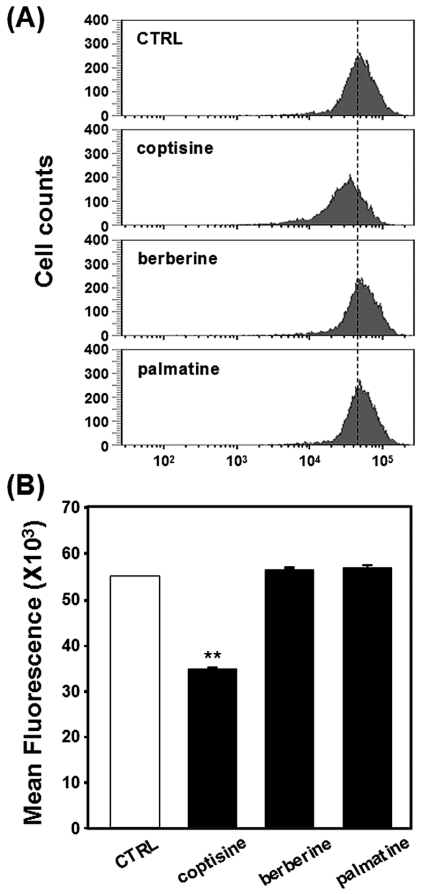

Fig. 3. Effects of Isoquinoline Alkaloids on the Mdr1a/1b Function in A10 Cells

A10 cells were seeded into 12-well plates at 3×10^4 cells/well, cultured for 48 h and then incubated with isoquinoline alkaloids at 30 μM. After 24 h, the medium was changed to DMEM without FCS, and the cells were cultured for 30 min. The medium was then replaced with DMEM containing 5 μM rhodamine 123 and the cells were incubated for 2 h. The cells were harvested and washed with HBSS, and the intracellular retention of rhodamine 123 was measured by flow cytometry. (A) Histograms of the intracellular rhodamine 123 intensities measured by flow cytometry. (B) Mean fluorescence intensities of the groups. Data are expressed as the means±S.D. of three wells from a representative of two independent experiments. ** $p<0.01$ *vs.* the CTRL group.

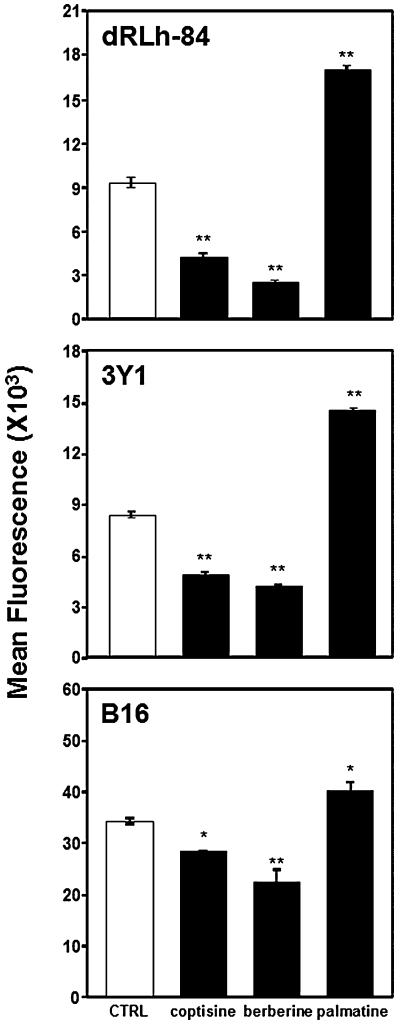

Fig. 4. Effects of Isoquinoline Alkaloids on the Mdr1a/1b Function in Various Cell Types

Cells were seeded into 12-well plates at specified concentrations (dRLh-84: 1.2×10^4 cells/well; 3Y1: 1.5×10^4 cells/well; B16: 2.5×10^4 cells/well), cultured for 48 h and then incubated with isoquinoline alkaloids at 30 μM. After 24 h, the medium was changed to DMEM without FCS, and the cells were cultured for 30 min. The medium was then replaced with DMEM containing 5 μM rhodamine 123 and the cells were incubated for 2 h. The cells were harvested and washed with HBSS, and the intracellular retention of rhodamine 123 was measured by flow cytometry. Data are expressed as the means±S.D. of three wells from a representative of two independent experiments. * $p<0.05$, ** $p<0.01$ *vs.* the CTRL group.

ferences among the three isoquinoline alkaloids for the regulation of Mdr1a/1b expression in VSMCs. In A10 cells, coptisine induced mRNA and protein expressions of Mdr1a and Mdr1b and stimulated Mdr1a/1b-mediated rhodamine 123 efflux from the cells. Meanwhile, berberine and palmatine slightly increased the mRNA levels of *Mdr1a* and *Mdr1b*, but failed to induce Mdr1a/1b protein expression or stimulate the efflux of intracellular rhodamine 123. These results suggest that coptisine selectively acts on A10 cells to regulate MDR1 function. In contrast to the findings for A10 cells, all three alkaloids were capable of inducing Mdr1a/1b protein expression in dRLh-84, 3Y1 and B16 cells. When the function of the induced Mdr1a/1b was examined, both coptisine and berberine stimulated the efflux of intracellular rhodamine 123 and decreased its intracellular retention, whereas palmatine did not. The currently accepted model of MDR1 function is as follows: the substrate directly interacts with the protein drug-binding pocket in MDR1 and is pumped into the extracellular space, assisted by hydrolysis of ATP to ADP.[25] Therefore, the lack of ability of palmatine to stimulate rhodamine 123 efflux was deduced by the finding that palmatine stimulated the ATPase activity inherent in Mdr1a as a substrate for Mdr1a more effectively than coptisine or berberine. In other words, palmatine competed with rhodamine 123 for Mdr1a/1b, resulting in a reduction in the rhodamine 123 efflux. Taken together, only coptisine can induce functional Mdr1a/1b in A10 cells, while berberine and palmatine are only unable to induce Mdr1a/1b in A10 cells.

MDR1 (Mdr1a/1b) is induced by not only a number of chemical compounds but also physical stresses, such as X-irradiation, ultraviolet light irradiation, serum starvation, heat shock and anticancer agents.[33] The actual mechanism of transcription of the *MDR1* gene is surprisingly complex, although the nuclear receptor pregnane X receptor, transcription factor Sp1 and nuclear factor Y-box binding protein 1 (YB-1) are likely to be involved in the activation of *MDR1* gene transcription.[34—36] YB-1 is a multifunctional regulator of gene expression associated with cell growth, drug resist-

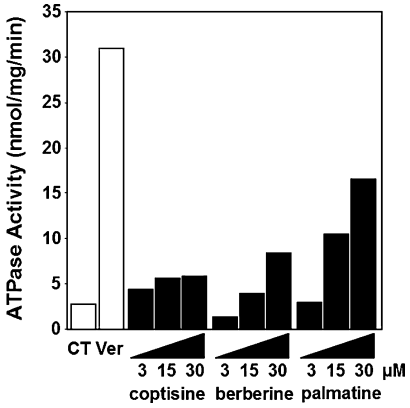

Fig. 5. Effects of Isoquinoline Alkaloids on the Vanadate-Sensitive ATPase Activity of Rat Mdr1a

Recombinant rat Mdr1a cell membranes (20 μg/well) and various concentrations of isoquinoline alkaloids were incubated for 3 min in the presence or absence of 300 μM sodium orthovanadate in 96-well plates. The reaction was initiated by the addition of 3 mM Mg-ATP and allowed to continue for 20 min. The reaction was stopped by the addition of 10% sodium dodecyl sulfate and the released inorganic phosphate was measured by photospectrometry. Data are expressed as the means of duplicate experiments.

ance and DNA synthesis to control transcription and translation positively or negatively, depending on the extent of YB-1 expression.[37] YB-1 is also transcribed in response to genotoxic stress and is translocated into the nucleus.[38] In addition, YB-1 can act as a poly(A)-binding protein, and stimulates and represses translation at low and high concentrations, respectively. Berberine and palmatine are well known to strongly interact with double-stranded DNA[39] and the poly(A) tails of RNAs,[40,41] whereas coptisine only weakly binds to double-stranded DNA.[42] However, in the cellular context, the aspects of whether berberine and palmatine cause DNA damage are still unclear. For example, some reports have indicated that berberine elicits DNA cleavage in cells, thereby leading to apoptosis,[43,44] while other reports have described that berberine protects against the DNA damage induced by hydrogen peroxide or peroxynitrite.[45,46] If isoquinoline alkaloids induce DNA or RNA damage in cells, the resulting genotoxic stress may be associated with YB-1 induction followed by MDR1 induction. However, the mechanism underlying the observation that only coptisine, but not berberine and palmatine, induces Mdr1a/1b in A10 cells is still unclear.

We previously reported that coptisine selectively prevents VSMC proliferation by blocking the cell cycle at the G_2/M and G_0/G_1 phases with a 50% growth inhibition (GI_{50}) value of 3.3 μM, compared with various cells such as 3Y1 fibroblasts, dRLh-84 hepatoma, B16 melanoma and HeLa cells (GI_{50} values of 35 μM to >140 μM).[16] In contrast, both berberine and palmatine did not show such potent and selective antiproliferative effects on VSMCs.[21] In our preliminary experiment, when the effect of coptisine on proliferation of A10 cells transfected with a siRNA for *Mdr1a/1b* was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the antiproliferative effect of coptisine remained unchanged regardless of the presence or absence of Mdr1a/1b proteins, the levels of which were determined by western blot analysis (unpublished data). The results suggest that Mdr1a/1b induction in A10 cells by coptisine appears not to be associated with antiproliferative effect on A10 cells shown by coptisine. Although the precise mechanism by which coptisine displays selective effect on VSMCs is required to be determined, the targeting molecule related to selective effect of coptisine is likely to exist in signaling pathway upstream of the molecules controlling Mdr1a/1b induction and cell cycle arrest. Given that berberine and palmatine were only unable to induce Mdr1a/1b in VSMCs and only had no antiproliferative effects on VSMCs,[16] VSMCs appear to lack the molecular targets that mediate the actions of berberine and palmatine or possess inhibitory molecules that block the actions of berberine and palmatine.

In conclusion, we have demonstrated that coptisine induced Mdr1a/1b in all cells examined, including VSMCs, and activated rhodamine 123 efflux, whereas berberine and palmatine failed to activate MDR1 function in VSMCs, thereby suggesting that coptisine possesses selective effects on VSMC functions as described in our previous reports.[16,21]

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