Reversing Effect of Agosterol A, a Spongean Sterol Acetate, on Multidrug Resistance in Human Carcinoma Cells

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The effect of agosterol A, a novel polyhydroxylated sterol acetate isolated from a marine sponge, on P-glycoprotein (P-gp)-mediated multidrug-resistant cells (KB-C2) and the multidrug resistance associated protein (MRP1)-mediated multidrug-resistant cells (KB-CV60) was examined. Agosterol A reversed the resistance to colchicine in KB-C2 cells and also the resistance to vincristine in KB-CV60 cells at 3 to 10 μM concentration. Agosterol A at 3 μM increased the vincristine concentration in both KB-C2 cells and KB-CV60 cells to the level in parental KB-3-1 cells. Agosterol A also decreased the efflux of vincristine from both KB-C2 cells and KB-CV60 cells to the level seen in KB-3-1 cells. Agosterol A inhibited the [^{3}H]azidopine-photolabeling of P-gp and also inhibited the uptake of [^{3}H]S-(2,4-dinitrophenyl)glutathione (DNP-SG) in inside-out membrane vesicles prepared from KB-CV60 cells. We conclude that agosterol A directly inhibited drug efflux through P-gp and/or MRP1.

Key words: Agosterol A — Multidrug resistance tumor — P-Glycoprotein — Multidrug resistance associated protein — Glutathione

Tumor cells which acquire drug resistance to several anticancer drugs having unrelated action mechanisms and chemical structures are called multidrug resistant (MDR), and are a major cause of failure of cancer chemotherapy. In many cases, MDR tumor cells acquire the resistance by overexpressing membrane proteins which excrete the antitumor drug. Overexpression of the transmembrane glycoprotein P-glycoprotein (P-gp), which belongs to a superfamily of adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters, has been observed in various MDR cell lines[1] and is closely related to drug resistance in clinical treatment.[2] On the other hand, non P-gp-mediated MDR[3] has been reported. MDR associated protein (MRP1), which is a 190 kDa transmembrane glycoprotein, was found in many non P-gp-mediated MDR tumor cells.[4] MRP1 is also a member of the ABC superfamily of membrane proteins and is an ATP-dependent glutathione-S conjugate export pump (GS-X pump), which transports glutathione conjugates (e.g., leukotriene C$_4$).[5]

Only a few reversing agents for MRP1-mediated MDR are known, while many compounds act as reversing agents (calcium channel blockers like verapamil, calmodulin inhibitor, cyclosporin A, etc.) for P-gp-mediated MDR.[6, 7] 2-[4-(Diphenylmethyl)-1-piperazinyl]-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide (PAK-104P), a pyridine analog, was found to completely reverse the resistance to vincristine in MRP1-overexpressing MDR cells.[8] 3-([{3-(2-[7-Chloro-2-quinolinyl]ethynyl)phenyl}-{(3-dimethylamino-3-oxopropyl)thio}methyl]thio)propanoic acid (MK571), a leukotriene C$_4$ receptor antagonist, was also shown to reverse MRP1-mediated MDR.[9] However, it is difficult to use the reversing agents for cancer chemotherapy because they were originally developed to show other biological effects. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, reversed the resistance to anti-tumor agents in MRP1-overexpressing MDR cells by reducing the intracellular glutathione content.[10]

In the course of our search for MDR-reversing substances in tumor cells, we isolated agosterol A, which reverses both P-gps and MRP1-mediated MDR, from a marine sponge of *Spongia* sp. and determined its absolute stereostructure.[11] Agosterol A is a novel polyhydroxylated sterol acetate, the chemical structure of which is quite different from those of known MDR-reversing agents. We isolated several related compounds from the same marine sponge and also prepared synthetic derivatives for study of the structure-activity relationship.[12] Furthermore, in order to access a simplified lead compound, we undertook the synthesis of 4-deacetoxyagosterol A.[13] In this paper, we present details of the biological evaluation of agosterol A.

MATERIALS AND METHODS

Materials Agosterol A and agosterol B were isolated from a marine sponge of *Spongia* sp. collected in Mie Pre-

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fecture, Japan, by bioassay-guided separation. Details of the isolation and the structure determination of agosterol A and its analogs were described previously (Fig. 1).[11, 12] 3-Deacetylagosterol A, 3,4,6-trideacetylagosterol A (IAG-A) and 22-dehydroxyagosterol A were synthesized from agosterol A (Fig. 1).[12] 11-Dehydroxyagosterol A was derived from agosterol C (Fig. 1).[12] [3H]Vincristine sulfate (5.70 Ci/mmol) was purchased from Amersham International Co., Ltd. (Buckinghamshire, UK). S-(2,4-Dinitrophenyl)-glutathione (DNP-SG) and [3H]DNP-SG were synthesized as described by Awasthi *et al.*[14] PAK-104P was kindly provided by Nissan Chemical Industries (Chiba). Vincristine, doxorubicin, colchicine and other drugs were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO).

Cell lines Human epidermoid carcinoma KB cells were subcloned twice, and a single recloned line, KB-3-1, was used as the parental cell line for the present study.[15] KB-3-1 cells were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) with 0.44 mg/ml of glutamine, 50 μg/ml of kanamycin sulfate, supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 at 37°C. A P-gp-mediated MDR mutant, KB-C2, was isolated from KB-3-1 cells and maintained in the medium with 2 μg/ml of colchicine.[15, 16] An MRP1-mediated MDR mutant, KB-CV60, was also isolated from KB-3-1 cells and maintained in the medium with 1 μg/ml of cepharanthine and 60 ng/ml of vincristine.[17]

Cell survival by MTT assay 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay was used as described in the previous paper[18] to assess the sensitivity of the cells to anti-tumor agents *in vitro*.

Drug accumulation and efflux The accumulation and efflux of vincristine were evaluated by modifications of the methods described previously.[8] To measure drug accumulation, confluent monolayers of KB-C2 cells or KB-CV60 cells were preincubated with or without MDR-reversing agents in RPM1 1640 medium for 15 min at 37°C and then treated with 1 μM [3H]vincristine for 1 h at 37°C. Cells were washed with ice-cold phosphate-buffered saline (PBS) three times, then solubilized with 1% Triton X-100 and 0.2% sodium dodecylsulfate (SDS) in 10 mM phosphate buffer (pH 7.4). The radioactivity of [3H]vincristine was measured in a liquid scintillation counter. To measure drug efflux, the cells (1×10^4) were preincubated for 72 h and further incubated with or without agosterol A for 15 min at 37°C. The medium was replaced with fresh medium, and the cells were treated with 1 μM [3H]vincristine in the presence or absence of agosterol A for 1 h at 37°C. The cells were washed once with PBS and then the medium was replaced with fresh medium. The cells were further incubated in the presence or absence of agosterol A at 37°C. The radioactivity of [3H]vincristine remaining in the cells was determined by using the same method as for measuring drug accumulation. Verapamil was used as a positive control.

Preparation of membrane vesicles Inside-out membrane vesicles were prepared from KB-C2 or KB-CV60 cells by means of the nitrogen cavitation method.[19] The cells were suspended in buffer A (10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and 0.2 mM $CaCl_2$) and equilibrated at 4°C for 30 min under a nitrogen pressure of 25 kg/cm². After cavitation, a final 1 mM concentration of ethylenediaminetetraacetic acid (EDTA) was added to the resulting suspension of lysed cells and the whole was diluted with buffer B (10 mM Tris-HCl (pH 7.4), and 0.25 M sucrose), then centrifuged at 1000g for 10 min at 4°C. The supernatant was layered onto a 35% sucrose cushion (10 mM Tris-HCl (pH 7.4), 35% sucrose and 1 mM EDTA) and centrifuged for 30 min at 16 000g at 4°C. The interface was collected, diluted with buffer B and centrifuged again for 45 min at 100 000g. The collected vesicle pellet was resuspended in buffer B by using a 25-gauge needle. The percentage of inside-out membrane vesicle formation[20] was about 50%. Protein concentration in the vesicles was determined by Bradford's method.[21] Vesicles were stored at −80°C.

Photoaffinity labeling of P-gp with [3H]azidopine A suspension of membrane vesicles prepared from KB-C2 cells (100 μg of protein) was incubated with 0.75 μM [3H]azidopine (53 Ci/mmol) for 15 min at room temperature in the presence or absence of MDR-reversing agents. After continuous irradiation at 366 nm for 20 min at 25°C,
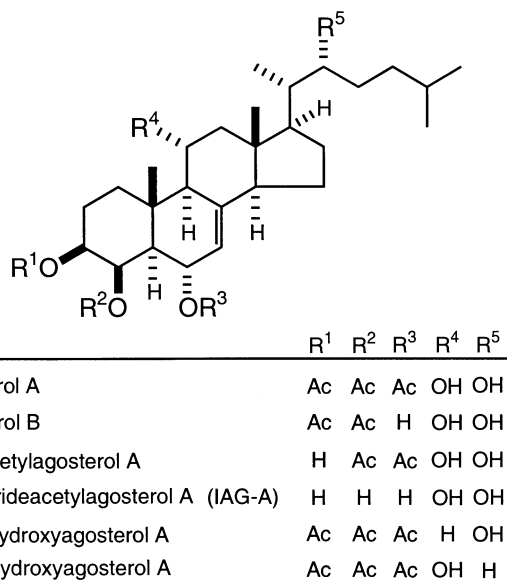

Fig. 1. Chemical structures of agosterols.

	R¹	R²	R³	R⁴	R⁵
agosterol A	Ac	Ac	Ac	OH	OH
agosterol B	Ac	Ac	H	OH	OH
3-deacetylagosterol A	H	Ac	Ac	OH	OH
3,4,6-trideacetylagosterol A (IAG-A)	H	H	H	OH	OH
11-dehydroxyagosterol A	Ac	Ac	Ac	H	OH
22-dehydroxyagosterol A	Ac	Ac	Ac	OH	H

the whole was dissolved in SDS buffer as described in the paper.[22]

SDS gel electrophoresis Samples labeled with [³H]azidopine were dissolved in 50 m*M* Tris-HCl at pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 5% glycerol, and 0.1% bromophenol blue solution. Electrophoresis on 7.5% SDS (w/v) polyacrylamide gels was carried out according to Laemmli's method without heating.[23]

Membrane vesicle transport ATP-dependent transport of DNP-SG into the membrane vesicles and its inhibition by reversing agents were measured by the method described by Ishikawa and Ali-Osman.[24] The membrane vesicles (25 μg of protein) were suspended in the medium (50 μl of final volume) containing 20 μ*M* [³H]DNP-SG, 0.25 *M* sucrose, 10 m*M* Tris-HCl (pH 7.4), 10 m*M* MgCl₂, 2 m*M* ATP, 10 m*M* phosphocreatine, and 100 μg/ml creatine phosphokinase with or without DNP-SG. The whole was kept at 37°C and the reaction was stopped by adding 3 ml of the ice-cold stop solution (0.25 *M* sucrose, 100 m*M* NaCl, and 10 m*M* Tris-HCl (pH 7.4)). The whole was passed through Millipore filters (GVWP: Millipore, Bedford, MA, 0.22 μm pore size) under light decompression. The residue was washed with 3 ml of the ice-cold stop solution three times and then dried at 50°C for 10 min. The radioactivity of each residue was measured with a liquid scintillation counter. In the control experiments, ATP was replaced with an equal concentration of adenosine 5′-monophosphate (5′-AMP). The net ATP-dependent transport was calculated by subtracting the value obtained in the presence of 5′-AMP from that obtained in the presence of ATP. To examine whether [³H]DNP-SG was actually transported to intravesicular space or bound to the vesicle membrane, the uptake of [³H]DNP-SG was measured with different concentrations of sucrose.

Measurement of intracellular glutathione concentration Confluent monolayers of KB-CV60 cells were harvested and washed with ice-cold PBS. The cells were suspended in 0.5 ml of 5% metaphosphoric acid solution and sonicated. The precipitated protein was removed by centrifugation (3000*g* for 10 min at 4°C) and the supernatant was used for glutathione measurement with a "BIOXYTECH" GSH-400 kit (Bonneuil/MARNE, Cedex, France).

Statistical analysis Differences between groups were tested by one-way ANOVA or Student's *t* test. Significance levels given are those for the two-tailed Student's paired *t* test. Data are presented as means±SE. Differences were considered to be significant when *P*<0.05.

RESULTS

Reversal of resistance in MDR tumor cells by agosterol A Fig. 2 shows the growth inhibition curves by anti-cancer agents in the presence or absence of MDR-reversing agents. Agosterol A (3 μ*M*) and verapamil (10 μ*M*) completely reversed the resistance to colchicine in KB-C2 cells. Agosterol A (3 μ*M*) also completely reversed the resistance to vincristine in KB-CV60 cells, while verapamil (10 μ*M*) showed only moderate effect. The sensitivity of the cells to vincristine, doxorubicin, and etoposide with or without reversing agents was also examined and the results are summarized in Table I. Agosterol A com-
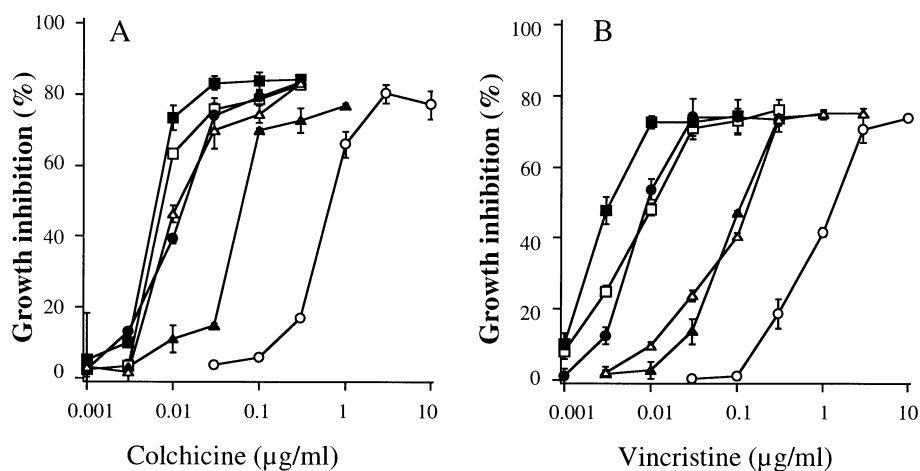

Fig. 2. Reversal of MDR to colchicine in KB-C2 cells (A) and to vincristine in KB-CV60 cells (B) by agosterol A. Sensitivity was measured in the absence or presence of various concentrations of agosterol A and verapamil. □, KB-3-1; ○, KB-C2 (A) or KB-CV60 (B); ■, KB-C2 (A) or KB-CV60 (B) + agosterol A (10 μ*M*); ●, KB-C2 (A) or KB-CV60 (B) + agosterol A (3 μ*M*); ▲, KB-C2 (A) or KB-CV60 (B) + agosterol A (1 μ*M*); △, KB-C2 (A) or KB-CV60 (B) + verapamil (10 μ*M*). Points represent means (±SE) of triplicate determinations.

Table I.   Reversal of MDR in KB-C2 Cells and KB-CV60 Cells by Agosterol A, Verapamil and BSO

Treatment	IC_{50} [a, e]		
	KB-3-1	KB-C2	KB-CV60
vincristine[c]	1.44±0.10 (1.00)[b]	142±10 (99)	125±7 (87)
+agosterol A (3 μM)	0.19±0.03 (0.13)	1.50±0.11 (1.21)	1.70±0.19 (1.18)
+agosterol A (1 μM)	0.41±0.03 (0.28)	16.5±0.4 (11.5)	2.37±0.16 (1.64)
+verapamil (10 μM)	0.36±0.06 (0.25)	2.27±0.36 (1.57)	14.5±0.3 (10.1)
+verapamil (3 μM)	0.71±0.07 (0.49)	14.7±0.2 (10.2)	44.0±1.6 (30.5)
+BSO (100 μM)	0.36±0.01 (0.25)	ND	7.93±0.97 (5.50)
doxorubicin[c]	12.5±0.2 (1.00)	1352±18 (108)	956±11 (76.5)
+agosterol A (3 μM)	5.63±0.33 (0.45)	13.4±0.8 (1.07)	8.33±0.51 (0.51)
+agosterol A (1 μM)	5.13±0.41 (0.41)	116±6 (9.28)	25.9±1.2 (0.67)
+verapamil (10 μM)	3.63±0.27 (0.29)	16.9±2.2 (1.35)	128±10 (10.2)
+verapamil (3 μM)	4.75±0.38 (0.38)	257±0 (20.6)	535±13 (42.8)
+BSO (100 μM)	4.38±0.62 (0.35)	ND	80.4±7.7 (6.43)
colchicine[c]	8.27±0.01 (1.00)	1020±87 (123)	27.4±1.8 (2.98)
+agosterol A (3 μM)	5.52±0.32 (0.66)	8.38±0.54 (1.01)	6.18±0.26 (0.74)
+agosterol A (1 μM)	5.84±0.48 (0.70)	91.3±6.4 (11.0)	8.88±1.51 (1.07)
+verapamil (10 μM)	5.73±0.38 (0.69)	15.8±0.6 (1.91)	13.3±0.7 (1.60)
+verapamil (3 μM)	6.07±0.23 (0.73)	154±10 (18.6)	19.6±0.7 (2.37)
+BSO (100 μM)	5.68±0.18 (0.68)	ND	10.5±0.8 (1.26)
etoposide[d]	8.09±0.35 (1.00)	143±11 (17.68)	55.0±2.8 (6.79)
+agosterol A (3 μM)	2.93±0.17 (0.36)	7.84±0.22 (0.96)	8.24±1.74 (1.01)
+agosterol A (1 μM)	3.88±0.09 (0.47)	24.8±0.4 (3.07)	12.6±1.2 (1.55)
+verapamil (10 μM)	1.69±0.23 (0.20)	13.8±2.1 (1.70)	22.8±1.2 (2.82)
+verapamil (3 μM)	3.22±0.51 (0.39)	48.1±4.4 (5.94)	27.6±1.7 (3.41)
+BSO (100 μM)	5.68±0.66 (0.70)	ND	14.9±2.3 (1.84)

a) Data represent means±SE of triplicate determinations from at least three separate experiments.
b) Relative resistance value (IC_{50} value of anti-tumor agent against KB-3-1 cells, KB-C2 cells, and KB-CV60 cells with or without reversing agent was divided by IC_{50} value of anti-tumor agent against KB-3-1 cells without reversing agent).
c) IC_{50} values of vincristine, doxorubicin, and colchicine are presented in nM.
d) IC_{50} value of etoposide is presented in μM.
e) Agosterol A (≤10 μM) and verapamil (≤10 μM) showed no cytotoxicity to KB-3-1, KB-C2, or KB-CV60 cells.
ND: not determined.

pletely reversed the resistance to vincristine, colchicine, doxorubicin, and etoposide in both KB-C2 cells and KB-CV60 cells at 3 μM concentration. We also examined the cytotoxicity of the MDR-reversing agents by MTT assay.

MDR-reversing effects of agosterol A and related compounds   The reversal of the resistance to vincristine by several analogs and synthetic derivatives of agosterol A was examined (Table II). Agosterol B lacking an acetyl group at C-6 completely reversed the resistance to vincristine in KB-CV60 cells at 10 μM concentration and also showed moderate activity in KB-C2 cells. 3-Deacetylagosterol A lacking an acetyl group at C-3 showed a moderate reversing effect in both KB-C2 cells and KB-CV60 cells

at 10 μM concentration. The reversing activity of agosterol B was 10-fold stronger than that of 3-deacetylagosterol A and the reversing activities of both compounds were much weaker than that of agosterol A. IAG-A, lacking all three acetyl groups in agosterol A, was slightly active in KB-CV60 cells and showed no activity in KB-C2 cells. 11-Dehydroxyagosterol A and 22-dehydroxyagosterol A, lacking a hydroxyl group at C-11 or C-22, showed moderate activity in KB-C2 cells and was slightly active in KB-CV60 cells at 10 μM concentration.

Effect of MDR-reversing agents on intracellular accumulation and efflux of [³H]vincristine   We examined the effect of agosterol A on the accumulation of vincristine in

Table II. Reversal of Resistance to Vincristine by Agosterol A and Its Derivatives

Treatment	IC$_{50}$ (nM) [a, c]		
	KB-3-1	KB-C2	KB-CV60
vincristine	1.44±0.10 (1.00) [b]	142±10 (98.6)	125±7 (86.8)
+agosterol A (3 μM)	0.19±0.03 (0.13)	1.50±0.11 (1.21)	1.70±0.19 (1.18)
+agosterol A (1 μM)	0.41±0.03 (0.28)	16.5±0.35 (11.5)	2.37±0.16 (1.64)
+agosterol B (10 μM)	0.30±0.02 (0.21)	9.75±0.95 (6.77)	0.84±0.04 (0.58)
+agosterol B (3 μM)	0.34±0.08 (0.23)	124±13 (86.1)	2.61±0.22 (1.81)
+agosterol B (1 μM)	0.49±0.01 (0.34)	183±20 (127)	56.1±6.4 (39.0)
+3-deacetylagosterol A (10 μM)	0.40±0.02 (0.27)	85.3±7.7 (59.3)	8.59±0.14 (5.97)
+3-deacetylagosterol A (3 μM)	0.79±0.01 (0.54)	136±4 (94.4)	30.9±7.3 (21.5)
+3-deacetylagosterol A (1 μM)	1.84±0.16 (1.27)	139±16 (96.5)	99.8±4.3 (69.3)
+IAG-A (10 μM)	0.14±0.01 (0.09)	143±11 (99.3)	51.0±1.9 (35.4)
+IAG-A (3 μM)	0.93±0.03 (0.64)	151±38 (104)	73.2±9.6 (50.8)
+IAG-A (1 μM)	2.16±0.14 (1.50)	162±11 (112)	147±11 (102)
+11-dehydroxyagosterol A (10 μM)	0.32±0.02 (0.22)	13.3±1.8 (9.24)	50.4±8.9 (35.0)
+11-dehydroxyagosterol A (3 μM)	0.90±0.12 (0.63)	65.8±9.9 (45.7)	120±7 (83.3)
+11-dehydroxyagosterol A (1 μM)	2.30±0.44 (1.59)	96.7±12.4 (67.1)	135±10 (93.7)
+22-dehydroxyagosterol A (10 μM)	0.51±0.01 (0.35)	19.7±2.5 (13.7)	78.4±4.2 (54.4)
+22-dehydroxyagosterol A (3 μM)	1.25±0.02 (0.86)	73.6±9.4 (51.1)	129±6 (89.6)
+22-dehydroxyagosterol A (1 μM)	1.34±0.16 (0.93)	111±11 (77.3)	128±9 (88.8)

a) Data represent means±SE of triplicate determinations from at least three separate experiments.

b) Relative resistance value (IC$_{50}$ value of anti-tumor agent against KB-3-1 cells, KB-C2 cells or KB-CV60 cells with or without reversing agent was divided by IC$_{50}$ value of anti-tumor agent against KB-3-1 cells without reversing agent).

c) Agosterol A, agosterol B, 3-deacetylagosterol A, IAG-A, 11-dehydroxyagosterol A, and 22-dehydroxyagosterol A showed no cytotoxic activity at 10 μM concentration.

KB-C2 cells or KB-CV60 cells to investigate the mechanism of overcoming the resistance to vincristine in KB-C2 cells and KB-CV60 cells. As shown in Fig. 3, the intracellular concentration of vincristine in KB-C2 cells and KB-CV60 cells was approximately 40% of that in parental KB-3-1 cells. The accumulation of vincristine in KB-C2 cells was restored by the addition of agosterol A (10 μM) or verapamil (10 μM) to about 80% of that in KB-3-1 cells (Fig. 3A). Agosterol A (3 μM) also restored the accumulation of vincristine in KB-CV60 cells to a level equal to that in KB-3-1 cells, whereas verapamil (10 μM) did not (Fig. 3B). IAG-A, which has no reversing effect on the resistance to anti-tumor agents in KB-C2 cells, also showed no effect on accumulation of vincristine in KB-C2. On the other hand, IAG-A was slightly effective in the accumulation of vincristine in KB-CV60 cells. Agosterol A and verapamil at 10 μM showed no effect on the accumulation of vincristine in KB-3-1 cells (data not shown).

We further examined the effect of agosterol A on the efflux of accumulated vincristine in KB-C2 cells and KB-CV60 cells. The time course of the release of the accumulated vincristine was observed for 120 min (Fig. 4, A and B). KB-C2 cells and KB-CV60 cells rapidly released the accumulated vincristine compared with KB-3-1 cells. After 120 min, 80% of the accumulated vincristine was effluxed from KB-C2 cells and KB-CV60 cells, whereas about 60–70% of the accumulated vincristine was retained in KB-3-1 cells. Both agosterol A and verapamil at 10 μM significantly inhibited the efflux of the accumulated vincristine in KB-C2 cells (Fig. 4A), while only agosterol A (10 μM) strongly inhibited the efflux of the accumulated vincristine in KB-CV60 cells (Fig. 4B).

Inhibition of [³H]azidopine-labeling of P-gp by agosterol A and verapamil The effect of agosterol A and verapamil on the [³H]azidopine-photolabeling of P-gp in membrane vesicles prepared from KB-C2 cells was exam-
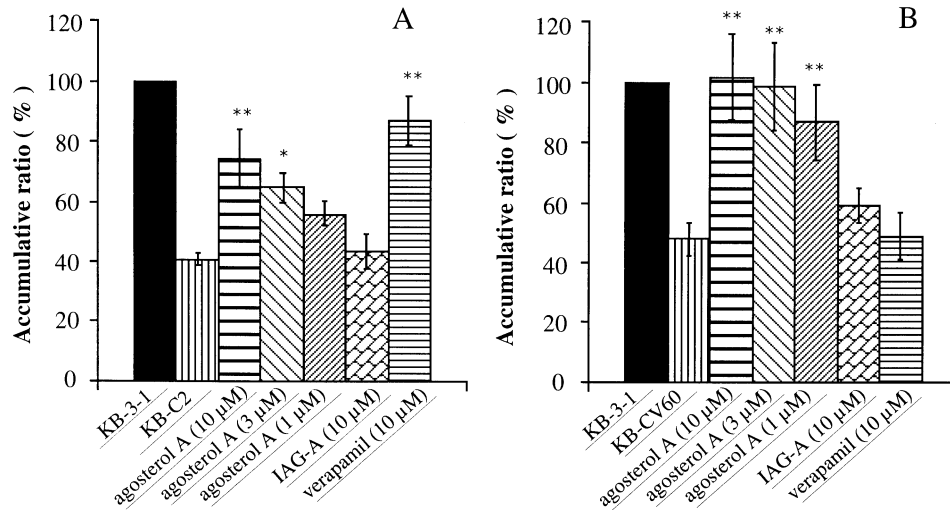
Fig. 3.   Effects of agosterol A, IAG-A, and verapamil on accumulation of vincristine in KB-C2 cells (A) and KB-CV60 cells (B). Vincristine accumulation in KB-C2 cells and KB-CV60 cells was measured after incubation with 1 μM [^{3}H]vincristine and 1 μM cold vincristine for 1 h without or with various concentrations of agosterol A, IAG-A, and verapamil. Vincristine amount accumulated in KB-3-1 cells is taken as 100%. Columns represent means (±SE) of triplicate determinations; ∗ $P<0.05$, ∗∗ $P<0.01$.
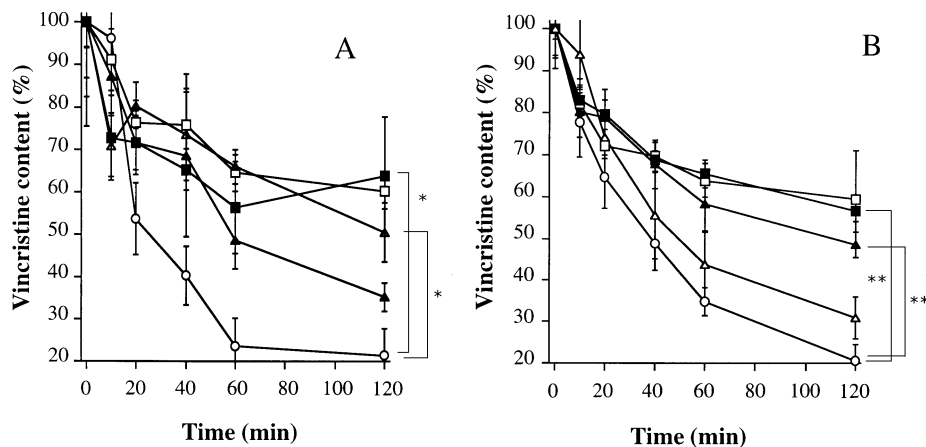
Fig. 4.   Efflux of accumulated vincristine from KB-3-1 cells, KB-C2 cells (A) and KB-CV60 (B). Vincristine content (%) after 1 h vincristine treatment in KB-C2 cells and KB-CV60 cells in the absence (○) or presence of 1 μM (▲) and 10 μM (■) agosterol A and 10 μM verapamil (△). Efflux of vincristine from KB-3-1 cells (□) was measured in the absence of reversing agent. Points represent means (±SE) of triplicate determinations; ∗ $P<0.05$, ∗∗ $P<0.01$.

ined. As shown in Fig. 5, agosterol A and verapamil inhibited the photolabeling by azidopine concentration-dependently. The inhibitory activity of agosterol A was much stronger than that of verapamil. On the other hand, IAG-A, which has no MDR-reversing effect in KB-C2 cells, did not inhibit the photolabeling.

Effect of agosterol A on ATP-dependent uptake of [^{3}H]DNP-SG into membrane vesicles prepared from KB-CV60 cells   To examine the effect of agosterol A on

the substrate efflux by MRP1, inside-out membrane vesicles were prepared from KB-CV60 cells, and DNP-SG uptake into membrane vesicles was measured in the presence or absence of MDR-reversing agents. DNP-SG is recognized as a good substrate for transport by MRP1.[25] Fig. 6 (A) shows the [^{3}H]DNP-SG uptake into membrane vesicles prepared from KB-CV60 cells in the presence of ATP. Agosterol A (10 to 100 μM) inhibited the [^{3}H]DNP-SG uptake concentration-dependently. PAK-104P (10 μM)

also showed inhibition of the [³H]DNP-SG uptake. IAG-A (100 μM) and BSO (100 μM) did not affect the DNP-SG uptake. The time course of [³H]DNP-SG accumulation in the membrane vesicles prepared from KB-3-1 cells and KB-CV60 cells is shown in Fig. 6 (B). ATP-dependent

[³H]DNP-SG uptake in membrane vesicles prepared from KB-CV60 cells showed linearity for about 2 min and reached a plateau in 10 min, while no ATP-dependent [³H]DNP-SG uptake in the membrane vesicles prepared from KB-3-1 cells was observed. Time-dependent accumulation of [³H]DNP-SG was strongly decreased by agosterol A (100 μM).

Effect of agosterol A and BSO on intracellular glutathione concentration The effects of agosterol A and BSO on the intracellular glutathione content were examined. As shown in Table III, glutatione content in KB-CV60 cells was 1.5-fold higher than that in KB-3-1 cells.
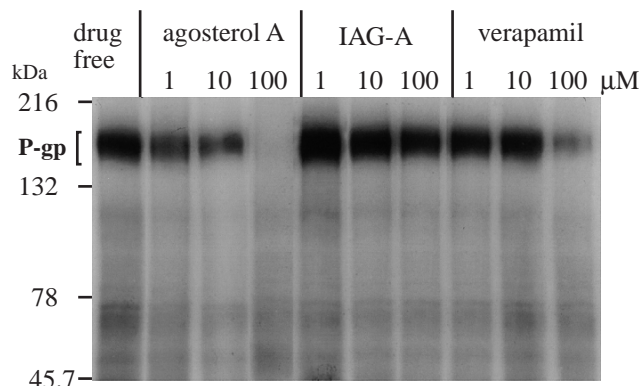

Fig. 5. Inhibition of [³H]azidopine-photolabeling of P-gp by agosterol A and verapamil. Membrane vesicles prepared from KB-C2 cells were incubated with [³H]azidopine in the absence or presence of agosterol A, IAG-A, and verapamil at various concentrations.

Table III. Effect of Agosterol A and BSO on Intracellular Glutathione Content in KB-3-1 Cells and KB-CV60 Cells

Treatment	Glutathione (nmol/10⁷ cells)	
	KB-3-1	KB-CV60
intact	76±21[a]	111±38
agosterol A (1 μM)	81±21	84±29
agosterol A (10 μM)	60±5.7	53±20
BSO (100 μM)	49±20	52±19

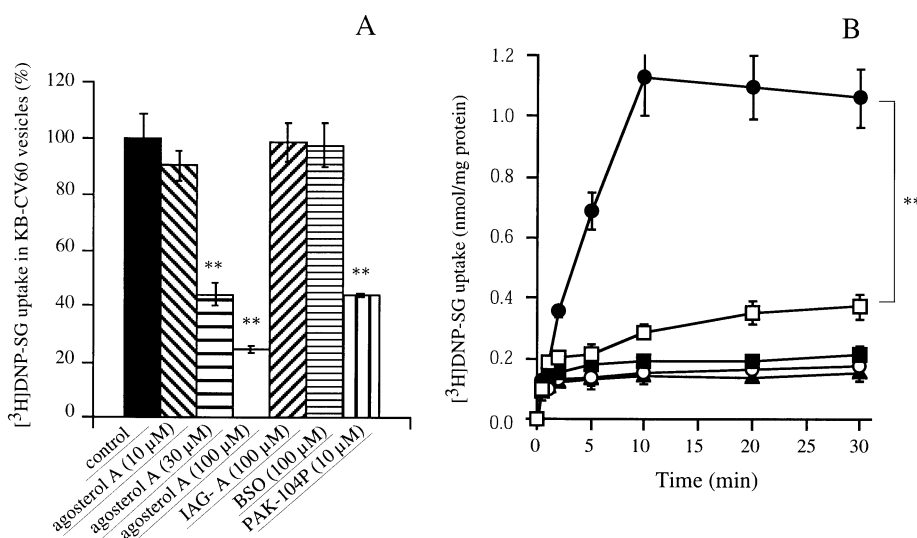a) Data represent means±SE of triplicate determinations from at least three separate experiments.

Fig. 6. Effect of agosterol A, IAG-A, and PAK-104P on ATP-dependent DNP-SG uptake (A). Membrane vesicles (25 μg protein) prepared from KB-CV60 cells were incubated at 37°C in 50 μl of transport buffer containing 20 nM [³H]DNP-SG in the presence of various concentrations of reversing agent. Columns represent means (±SE) of triplicate determinations; ∗ $P<0.05$, ∗∗ $P<0.01$. Time course of [³H]DNP-SG accumulation in membrane vesicles (B). Membrane vesicles (25 μg protein) prepared from KB-3-1 cells (○) and KB-CV60 cells (●) were incubated at 37°C in 50 μl of transport buffer containing 20 μM [³H]DNP-SG. To examine the effect of agosterol A, membrane vesicles prepared from KB-CV60 cells were incubated in the presence of 100 μM agosterol A (□). In control experiments, ATP was replaced with an equal concentration of 5′-AMP (KB-3-1, ▲ ; KB-CV60, ■). Points represent the means (±SE) of three separate experiments.

When KB-CV60 cells were treated with BSO (100 μM), glutathione content in KB-CV60 cells decreased to 50% of that in the absence of BSO. Agosterol A (1 and 10 μM) also decreased glutathione content in KB-CV60 cells to 80% and 50%, respectively, and the effect of agosterol A was 10-fold stronger than that of BSO.

DISCUSSION

The appearance of tumor resistance to anti-cancer drugs is a serious problem in cancer chemotherapy. Increased expression of P-gp and/or MRP1 in human tissues and cancers is also often detected.[1, 10, 25] A number of substances reversing P-gp-mediated MDR are known. However, most of them have side effects because they were actually developed as drugs with different action. Therefore, a new type of substance, which specifically inhibits the active efflux of anti-cancer drugs by P-gp and/or MRP1, is required in the clinical field.

The results in Table I and Fig. 2 demonstrate that agosterol A completely reversed the resistance to colchicine, vincristine, doxorubicin, and etoposide in KB-C2 cells and the resistance to vincristine, doxorubicin, and etoposide in KB-CV60 cells at 3 to 10 μM, respectively. This finding suggests that agosterol A reversed both P-gp and MRP1-mediated MDR. We have already reported that PAK-104P reversed both P-gp and MRP1-mediated MDR.[8] The reversing activity of agosterol A was almost equal to that of PAK-104P. As shown in Fig. 3 and Fig. 4, agosterol A inhibited the ATP-dependent active efflux of vincristine in both KB-C2 cells and KB-CV60 cells by increasing intracellular vincristine. Agosterol A showed a greater effect in enhancing the accumulation of vincristine in KB-CV60 cells than in KB-C2 cells. Agosterol A also inhibited the efflux of vincristine from KB-CV60 cells more strongly than that from KB-C2 cells. As shown in Fig. 5, agosterol A was found to inhibit the [^{3}H]azidopine-photolabeling of P-gp in membrane vesicles prepared from KB-C2 cells, whereas IAG-A, an inactive derivative of agosterol A, showed no inhibition. The ATP-dependent DNP-SG uptake into membrane vesicles prepared from KB-CV60 cells was also inhibited by agosterol A, while IAG-A showed no inhibitory activity (Fig. 6). These findings indicate that agosterol A directly interacted with P-gp and MRP1 to inhibit the efflux of anticancer agents.

MRP1 is an ATP-dependent transmembrane glycoprotein, which transports glutathione conjugates in a number of human tumor cells.[5, 26] BSO inhibits γ-glutamylcysteine synthetase, which is the rate-limiting enzyme in the glutathione biosynthetic pathway.[27] Compounds which down-regulate intracellular glutathione, such as BSO and acro-

lein, have been found to reverse MRP1-mediated drug resistance.[18, 28] We found that agosterol A also reduced the intracellular glutathione content in KB-CV60 cells, as shown in Table III. Agosterol A might have a dual function to reverse MRP1-mediated MDR by direct action on MRP1 and to down-regulate intracellular glutathione.

To clarify the structure-activity relationship of agosterol A, we examined the reversing activities of several derivatives of agosterol A, as shown in Table II. Agosterol A contains three acetyl and two hydroxyl groups. Each derivative, which lacks one acetyl (C-3 or C-6 position) or one hydroxyl (C-11 or C-22 position) group compared with agosterol A, showed weaker activity, while IAG-A lacking all three acetyl groups (C-3, 4, 6 position), showed little activity in KB-C2 and KB-V60 cells. Thus, all the functional groups are important for potent reversing activity. It is interesting to note that the derivatives lacking one acetyl group showed stronger reversing activity in KB-CV60 cells than in KB-C2 cells, and the derivatives lacking one hydroxyl group showed stronger activity in KB-C2 cells than in KB-CV60 cells. The acetoxyl groups in agosterol A might have a role in reversing P-gp-mediated drug resistance, while the hydroxyl groups might be important for reversing MRP1-mediated drug resistance.

Agosterol A is a novel polyhydroxylated sterol acetate, which is structurally unrelated to known MDR-reversing agents. We demonstrated that agosterol A enhanced the susceptibility to anti-tumor agents such as vincristine, colchicine, doxorubicin, and etoposide in P-gp- and MRP1-overexpressing MDR cells by inhibiting ATP-dependent drug efflux by P-gp and MRP1. The reduction of the intracellular glutathione content in KB-CV60 cells by agosterol A also favors reversal of MDR. Agosterol A is the first example of an agent which inhibits the function of both P-gp and MRP1 by direct interaction and also increases sensitivity to anti-tumor agents in MRP1-overexpressing MDR cells by reducing the intracellular glutathione content. Our previous study demonstrated that the combination of BSO and PAK-104P resulted in synergistic reversal of MDR.[29] Therefore, agosterol A might be a promising reversing agent to overcome both P-gp- and MRP1-mediated MDR of tumor cells.

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