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Reversal of P-Glycoprotein-mediated Paclitaxel Resistance by New Synthetic Isoprenoids in Human Bladder Cancer Cell Line

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We isolated a paclitaxel-resistant cell line (KK47/TX30) from a human bladder cancer cell line (KK47/WT) in order to investigate the mechanism of and reversal agents for paclitaxel resistance. KK47/TX30 cells exhibited 700-fold resistance to paclitaxel and cross-resistance to vinca alkaloids and topoisomerase II inhibitors. Tubulin polymerization assay showed no significant difference in the ratio of polymerized α- and β-tubulin between KK47/WT and KK47/TX30 cells. Western blot analysis demonstrated overexpression of P-glycoprotein (P-gp) and lung resistance-related protein (LRP) in KK47/TX30 cells. Drug accumulation and efflux studies showed that the decreased paclitaxel accumulation in KK47/TX30 cells was due to enhanced paclitaxel efflux. Cell survival assay revealed that verapamil and cepharanthine, conventional P-gp modulators, could completely overcome paclitaxel resistance. To investigate whether new synthetic isoprenoids could overcome paclitaxel resistance, we synthesized 31 isoprenoids based on the structure of *N*-solanesyl-*N*,*N*′-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB), which could reverse multidrug resistance (MDR), as shown previously. Among those examined, *trans*-*N*,*N*′-bis(3,4-dimethoxybenzyl)-*N*-solanesyl-1,2-diaminocyclohexane (N-5228) could completely reverse paclitaxel resistance in KK47/TX30 cells. N-5228 inhibited photoaffinity labeling of P-gp by [^{3}H]azidopine, suggesting that N-5228 could bind to P-gp directly and could be a substrate of P-gp. Next, we investigated structural features of these 31 isoprenoids in order to determine the structural requirements for the reversal of P-gp-mediated paclitaxel resistance, suggesting that the following structural features are important for overcoming paclitaxel resistance: (1) a basic structure of 8 to 10 isoprene units, (2) a cyclohexane ring or benzene ring within the framework, (3) two cationic sites in close proximity to each other, and (4) a benzyl group with 3,4-dimethoxy functionalities, which have moderate electron-donating ability. These findings may provide valuable information for the development of P-gp-mediated MDR-reversing agents.

Key words: Synthetic isoprenoid — Multidrug resistance — Bladder cancer

Several new chemotherapeutic agents have become available during the last decade. One such agent, paclitaxel, is reported to be effective for the treatment of patients with cancers of the lung, breast, ovary, brain, prostate, kidney and bladder.[1–7] However, drug resistance to paclitaxel, either natural or acquired, becomes a major obstacle to effective chemotherapy. Previous studies reported that mechanisms of paclitaxel resistance include P-glycoprotein (P-gp) overexpression,[8] and molecular alterations of microtubule genes and tumor suppressor genes, including *p53*.[9] P-gp-mediated paclitaxel resistance has been investigated in some cancer cell lines.[10–12] P-gp is believed to function as an energy-dependent efflux pump.[13] P-gp-mediated multidrug resistance (MDR) was shown to be reversed by a variety of compounds, such as calcium channel blockers, calmodulin antagonists, anthracycline and vinca alkaloid analogues, cyclosporines, dipyridamole and other hydrophobic, cationic compounds.[14]

Bladder cancer is the fourth most common cancer in males in the United States, being associated with 10 600 deaths annually.[15] Recently, paclitaxel has been shown to be effective in the treatment of advanced bladder cancer.[7] However, in human bladder cancer, little information is available on reversing agents and the mechanism of paclitaxel resistance. In the present study, we established KK47/TX30 paclitaxel-resistant cell line from the KK47/WT human bladder cancer cell line[16] and characterized it. Our study demonstrated that KK47/TX30 cells were approximately 700-fold resistant to paclitaxel and overexpressed P-gp and lung resistance-related protein (LRP). LRP is the major vault protein in human cell lines.[17] Vaults are cytoplasmic ribonucleoprotein organelles,[18] but

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5% of vaults are localized in nuclear pore complexes[19] and are associated with nucleocytoplasmic transport.[20] Only one report has previously demonstrated that LRP overexpression is associated with paclitaxel resistance.[21] However, the role of LRP in paclitaxel resistance remains unclear.

We previously reported that vitamin A, which has four isoprene units (geranylgeranyl), could potentiate the antitumor effect of some anticancer agents.[22] We also reported that *N*-solanesyl-*N*,*N*′-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB), with 9 isoprene units and a verapamil-like structure, could completely reverse MDR in cultured MDR cells without calcium-blocking activity.[23, 24] Akiyama *et al.*[25] demonstrated that SDB was a substrate of P-gp by photoaffinity labeling with [^{125}I]SDB. However, they did not establish what are the key structural features of SDB.[26]

We have now screened for potent reversing agents of paclitaxel resistance among 31 new isoprenoids synthesized based on SDB structure. We also investigated the structural features of isoprenoids required for activity to reverse paclitaxel resistance in order to facilitate development of more potent reversing agents. In the present study, we discuss the underlying mechanisms of paclitaxel resistance and the required structural features to reverse paclitaxel resistance in human bladder cancer cells.

MATERIALS AND METHODS

Cell lines and cell culture The multidrug-resistant mutant cell strain KK47/TX30 was selected in the presence of 0.75 μM paclitaxel. Cells were grown in monolayer culture in minimal essential medium (MEM) (Sigma-Aldrich Co., St. Louis, MO) containing 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX), 50 μg/ml streptomycin, 50 units/ml penicillin, and 2 m*M* L-glutamine, as described previously.[27]

Cell survival by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay Chemosensitivity *in vitro* was measured by means of MTT colorimetric assay performed in 96-well plates.[28] Equal numbers of cells (5×10^3 for KK47/WT and 8×10^3 for KK47/TX30) were incubated in each well with 180 μl of culture medium. After 24-h incubation (37°C with 5% CO_2), 20 μl of chemotherapeutic agent solution with or without reversing agent was added to the cultures and incubation was continued for 72 h. Thereafter, 50 μl of 1 mg/ml MTT in phosphate-buffered saline (PBS) (in g/liter: NaCl, 8.0; Na_2HPO_4, 1.15; KCl, 0.2; KH_2PO_4, 0.2) was added to each well and incubation was continued for an additional 4 h. The resulting formazan was dissolved in 100 μl of dimethylsulfoxide after aspiration of the culture medium. Plates were placed on a plate shaker for 5 min and read immediately at 570 nm using a Micro Plate Reader MPR-A4i (Tosoh, Tokyo). Cell survival was expressed as the paclitaxel concentration that inhibited the cell growth by 50% (IC_{50}) with or without isoprenoid. Cell viability was expressed in terms of the ratio of cellular cytotoxicity with and without isoprenoid.

Preparation for tubulin polymerization assay To quantitate tubulin polymerization, a simple assay was developed by modifying a method originally described by Ohta *et al.*[12] and Minotti *et al.*[29] Cells grown to confluence were washed twice with PBS, collected and adjusted to 1×10^6 cells/ml. One milliliter of each cell suspension was transferred to 1.5-ml Eppendorf tubes, centrifuged at 200*g* for 5 min and lysed at room temperature for 5 min with 1 ml of hypotonic buffer (1 m*M* $MgCl_2$, 2 m*M* ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5% Nonidet P-40, 2 m*M* phenylmethylsulfonyl fluoride, 15 mg/ml aprotinin, 20 m*M* Tris-HCl, pH 6.8 and 4 μg/ml paclitaxel). Samples were vigorously vortexed, then centrifuged at 14 000 rpm for 10 min at room temperature. The supernatants containing soluble (cytosolic) tubulin were transferred to another Eppendorf tube. The pellets, containing polymerized (cytoskeletal) tubulin, were resuspended in 1 ml of hypotonic buffer.

Membrane vesicle preparation Membrane vesicles were prepared as described previously[30] from cells grown in 24.5×24.5 cm tissue culture plates (NUNC, Roskilde, Denmark). Protein concentrations were determined according to the method of Bradford.[31]

Immunoblotting To investigate transporter proteins, membrane vesicles (100 μg protein) were mixed with sodium dodecyl sulfate (SDS) sample buffer (25 m*M* Tris-HCl, pH 6.8, 1% SDS, 4% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) and applied on 7.5% SDS-polyacrylamide gels without heating. To investigate suitable conditions for tubulin polymerization assay, the cytosolic and cytoskeletal fractions were each mixed with SDS sample buffer and heated at 95°C for 5 min. Twenty microliters of each sample was applied on 10% SDS-polyacrylamide gels. Then, the samples were separated by SDS-polyacrylamide gel electrophoresis[32] and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) electrophoretically for 36 min at 15 V[33] using a Transblot SD apparatus (BioRad, Richmond, CA) as described by Kyhse,[34] except that the buffer consisted of 48 m*M* Tris and 39 m*M* glycine containing 20% (v/v) methanol, pH 9.2.[35] After transfer, the membranes were blocked in washing buffer (0.35 *M* NaCl, 10 m*M* Tris-HCl, pH 8.0, and 0.05% Tween 20) containing 3% skim milk for 2 h at room temperature, followed by an overnight incubation with 1000-fold diluted antibody against P-gp, LRP, multidrug resistance-associated protein 1 (MRP1), canalicular multispecific organic anion transporter (cMORT)/MRP2, breast cancer resistance protein (BCRP), α-tubulin and β-tubulin in washing buffer containing 3% skim milk. The membranes were

washed three times with washing buffer and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG. PVDF membranes were rinsed once for 15 min and three times for 5 min with washing buffer and then evenly coated using the enhanced chemiluminescence (ECL) western blotting detection system (Amersham, Arlington Heights, IL) for 1 min. The membrane was immediately exposed to Kodak XAR film at room temperature for various periods in a film cassette. Bands were quantitated by densitometry. The ratio of polymerized tubulin was determined by dividing the densitometry value of polymerized tubulin by the total tubulin content (the sum of the densitometry values of soluble and polymerized tubulin).

Drug accumulation To measure drug accumulation, confluent monolayers of KK47/WT and KK47/TX30 cells in 24-well plates were incubated in 1 ml of serum-free MEM with or without *trans-N,N′*-bis(3,4-dimethoxybenzyl)-*N*-solanesyl-1,2-diaminocyclohexane (N-5228) at the indicated concentrations for 1 h at 37°C. Serum-free MEM (1 ml) containing 8.5 n*M* [^{3}H]paclitaxel and 1 μM cold paclitaxel with or without N-5228 was then added and incubation was continued for the indicated time periods. Cells were then washed three times with ice-cold PBS and harvested with trypsin treatment. Cellular pellets were suspended in 0.9 ml of H_2O and mixed thoroughly with 7 ml of Scintisol (Nakalai Tesque, Inc., Kyoto), and the radioactivity was counted.

Drug efflux To measure drug efflux, confluent monolayers of KK-47/WT and KK47/TX30 cells in 6-well plates were incubated in 2 ml of medium-A (serum- and glucose-free MEM containing 50 m*M* deoxyglucose and 15 m*M* sodium azide) with or without N-5228 at the indicated concentrations for 1 h at 37°C. Thereafter, 2 ml of medium-A containing 8.5 n*M* [^{3}H]paclitaxel and 1 μM cold paclitaxel with or without N-5228 was added and incubation was continued for 1 h. Cells were then washed once with ice-cold PBS and the efflux was followed over 60 min in medium-B (serum-free and 1 g/liter glucose MEM) with or without N-5228. At the indicated times, cells were washed three times with ice-cold PBS and harvested, and the radioactivity was counted.

Photoaffinity labeling Membrane vesicles (100 μg protein) were incubated with 0.75 μM [^{3}H]azidopine for 15 min at room temperature in the presence or absence of N-5228 in a 96-well plate. After continuous irradiation at 366 nm for 30 min at 25°C, samples were solubilized in SDS sample buffer, as described by Debenham *et al.*[36] Samples labeled with [^{3}H]azidopine were electrophoresed by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Subsequently, the gel was soaked in "Enlightning" (Biotechnology Systems, Boston, MA) for 15 min, dried, and exposed to Kodak XAR film at −80°C for 5 days.

Chemicals Isoprenoids were synthesized and kindly supplied by the Nissin Flour Milling Co. (Saitama) and Tohoku Pharmaceutical University (Sendai). The synthetic procedures are described in United States patents No. 5756475 and No. 6011069.[37, 38] A monoclonal antibody against Chinese hamster P-gp (C219), which was originally isolated by Kartner *et al.*,[39] was obtained from Alexis Co. (San Diego, CA). A monoclonal antibody against α- and β-tubulin was obtained from Sigma-Aldrich Co. A monoclonal antibody against human MRP1 (m6) was also obtained from Alexis Co. The polyclonal antibody against LRP[17] was kindly provided by Dr. S. Akiyama (Institute for Cancer Research, Faculty of Medicine, Kagoshima University). The polyclonal antibody against cMORT/MRP2 was provided by Dr. M. Kuwano (Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, Fukuoka). The polyclonal antibody against BCRP was provided by Dr. Y. Sugimoto (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo). Cepharanthine was from Kaken Pharmaceutical Co., Osaka. [^{3}H]Paclitaxel and [^{3}H]azidopine were obtained from Moravek Biochemicals, Inc. (Brea, CA) and Amersham, respectively. Other agents were purchased from Sigma-Aldrich Co.

RESULTS

Characteristics of parental and paclitaxel-resistant cell lines The IC_{50} of KK47/TX30 cells was 4646 n*M* and the relative resistance, which was expressed as the ratio of the IC_{50} concentration for KK47/TX30 versus KK47/WT cells, was 702-fold in the presence of paclitaxel by the MTT assay (Table I). The doubling times for KK47/WT and KK47/TX30 were 24.5 and 28.5, respectively.

Cross-resistance of KK47/TX30 cells We examined the

Table I. Drug-resistance Phenotype in KK47/WT and KK47/TX30 Cells

Chemotherapeutic agent	IC_{50} (n*M*)[a]		Ratio[b]
	KK47/WT	KK47/TX30	
Paclitaxel	6.62±0.47	4646±115	702
VLB	0.60±0.04	191±10.0	318
VCR	8.20±0.26	2195±128	268
ADM	83.5±1.74	830±50.1	10
VP-16	79.7±3.94	1340±562	17
MITO	399±15.8	2312±232	6
CDDP	4823±1290	4747±513	1
CPT	96.5±20.0	91.2±9.57	1

a) Cell survival was determined by MTT assay and expressed as the paclitaxel concentration that inhibited the cell growth by 50% (IC_{50}) with or without isoprenoid. Values are mean±SEM of triplicate determinations.
b) Relative resistance to KK47/WT cells.

relative resistance of KK47/TX30 cells to several agents with different mechanisms of action by means of the MTT assay. The IC_{50} values and the relative resistance are presented in Table I. Marked cross-resistance, by 318-fold and 268-fold, was demonstrated with vinca alkaloids, including vinblastine (VLB) and vincristine (VCR), respectively. There was also moderate cross-resistance (by 5- to 20-fold) to topoisomerase II inhibitors, such as doxorubicin (ADM), etoposide (VP-16) and mitoxantrone (MITO). However, no cross-resistance was observed for cisplatin (CDDP) and camptothecin (CPT).

Tubulin polymerization assay Since paclitaxel promotes tubulin polymerization, we compared the ratio of polymerized α- and β-tubulin between the parental KK47/WT and the resistant KK47/TX30 cells. The ratio of polymerized α- and β-tubulin was 75.4% and 71.2% in KK47/WT cells, respectively (Fig. 1). In KK47/TX30 cells, the ratio of polymerized α- and β-tubulin was 73.8% and 72.2%, respectively (Fig. 1). There was no significant difference in the ratio of polymerized α- and β-tubulin between KK47/WT and KK47/TX30 cells.

Immunoblot analysis for P-gp and LRP in KK47 cell lines P-gp and LRP expression in KK47 cells was investigated by immunoblots with a monoclonal antibody against P-gp (C219) and a polyclonal antibody against LRP, respectively. KB-C2, which is an MDR cell line, was used as the positive control for P-gp. P-gp was overexpressed in KK47/TX30 membrane vesicles, but not in KK47/WT (Fig. 2). SW620 human colon cancer cells (positive control for LRP) were incubated with sodium butyrate to increase LRP expression,[21] and the cytosolic fractions of SW620 cells were used. In cytosolic fractions, LRP was expressed highly in KK47/TX30, but weakly in KK47/WT. Furthermore, in nuclear extractions, LRP was also highly expressed in KK47/TX30, but not in KK47/WT (Fig. 2). To investigate other non-P-gp-mediated MDR in

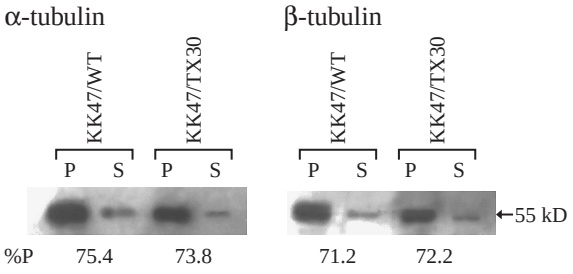

Fig. 1. Lack of significant differences in α- and β-tubulin contents between KK47/WT and KK47/TX30 cells. The ratio of polymerized α- and β-tubulin was 75.4% and 71.2%, respectively, in KK47/WT cells. In KK47/TX30 cells, the ratio of polymerized α- and β-tubulin was 73.8% and 72.2%, respectively. There was no significant difference in the ratio of polymerized α- and β-tubulin between the parental KK47/WT and the resistant KK47/TX30 cells. %*P*=percentage of polymerized tubulin.

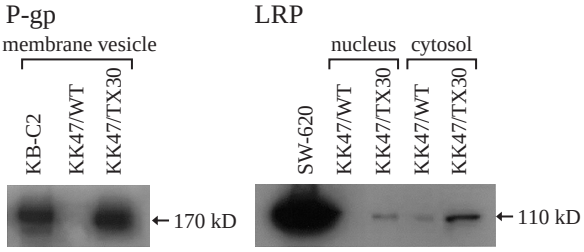

Fig. 2. Immunoblot analysis for P-gp in KK47 cells. P-gp was overexpressed in KK47/TX30 membrane vesicles, but not in KK47/WT.

Table II. Effects of MDR-reversing Agents on Paclitaxel Resistance

Treatment	IC_{50} (n*M*)[a]			
	KK47/WT	Ratio[b]	KK47/TX30	Ratio
Paclitaxel	6.62±0.47	1	4646±115	702
+Verapamil (5 *μM*)	1.74±0.08	0.26	43.7±2.10	6.60
+Verapamil (10 *μM*)	1.39±0.24	0.21	10.5±0.17	1.59
+Cepharanthine (5 *μM*)	1.41±0.09	0.21	16.7±0.89	2.52
+Cepharanthine (10 *μM*)	0.93±0.14	0.14	2.17±0.58	0.32
+N-5228 (5 *μM*)	6.51±0.27	0.98	1420±46	214
+N-5228 (10 *μM*)	6.51±0.45	0.98	273±20	41.2
+N-5228 (20 *μM*)	6.12±0.73	0.92	6.39±0.22	0.97
+N-5228 (30 *μM*)	5.69±0.26	0.86	5.21±1.12	0.79

a) Cell survival was determined by MTT assay and expressed as the paclitaxel concentration that inhibited the cell growth by 50% (IC_{50}) with or without isoprenoid. Values are mean±SEM of triplicate determinations.

b) Relative resistance to KK47/WT without the reversing agents.

KK47/TX30 cells, we also performed immunoblots using antibodies against MRP1, cMORT/MRP2 and BCRP, but no bands were detected in membrane vesicles prepared from any of the cells (data not shown).

Paclitaxel resistance in KK47/WT and KK47/TX30 cells by MDR-reversing agents and N-5228 N-5228, a new synthetic isoprenoid (structure shown in Fig. 5 and Table III), verapamil and cepharanthine were examined for the ability to reverse paclitaxel resistance in KK47/TX30 cells (Table II). We also examined the cytotoxic effect of the reversing agents by the MTT assay. N-5228 (20 μM), verapamil (10 μM) and cepharanthine (10 μM) had no cytotoxic effect on KK47/WT and KK47/TX30 cells. The IC_{50} of paclitaxel with or without the reversing agents was determined by MTT assay. At the concentration of 20 μM, N-5228 completely reversed paclitaxel resistance in KK47/TX30 cells, as did verapamil and cepharanthine (Table II). At lower concentration (5 μM or 10 μM) than 20 μM, N-5228 partially reversed paclitaxel resistance in the resistant cells (Table II).

Effect of N-5228 on cellular accumulation of [^{3}H]paclitaxel To investigate how N-5228 could completely reverse paclitaxel resistance in KK47/TX30 cells, we examined the effect of N-5228 on the accumulation of paclitaxel in KK47/WT and KK47/TX30 cells (Fig. 3A). The intracellular concentration of paclitaxel in KK47/TX30 cells remained at a low level until 120 min, whereas that in KK47/WT cells was approximately 25 times that in KK47/TX30 cells at 60 min. The addition of N-5228 (20 μM) enhanced paclitaxel accumulation in KK47/TX30 cells to levels similar to those in KK47/WT cells without N-5228. N-5228 (20 μM) enhanced paclitaxel accumulation in KK47/WT cells by 1.1-fold at 60 min.

Effect of N-5228 on efflux of [^{3}H]paclitaxel We investigated whether increased accumulation of paclitaxel in KK47/TX30 cells by N-5228 was due to inhibition of paclitaxel efflux. The remaining intracellular radioactivity of [^{3}H]paclitaxel was counted as a function of time after 1-h [^{3}H]paclitaxel accumulation (Fig. 3B). Efflux of more than 80% of intracellular paclitaxel was noted in KK47/TX30 cells, whereas the efflux was less than 30% in KK47/WT cells at 10 min after the start of the assay. At 30 min, more than 90% of intracellular paclitaxel had left KK47/TX30 cells, whereas more than 60% was retained in KK47/WT cells. However, N-5228 (20 μM) completely inhibited paclitaxel efflux in KK47/TX30 cells and increased the intracellular paclitaxel level to that observed in KK47/WT cells. N-5228 (20 μM) slightly inhibited

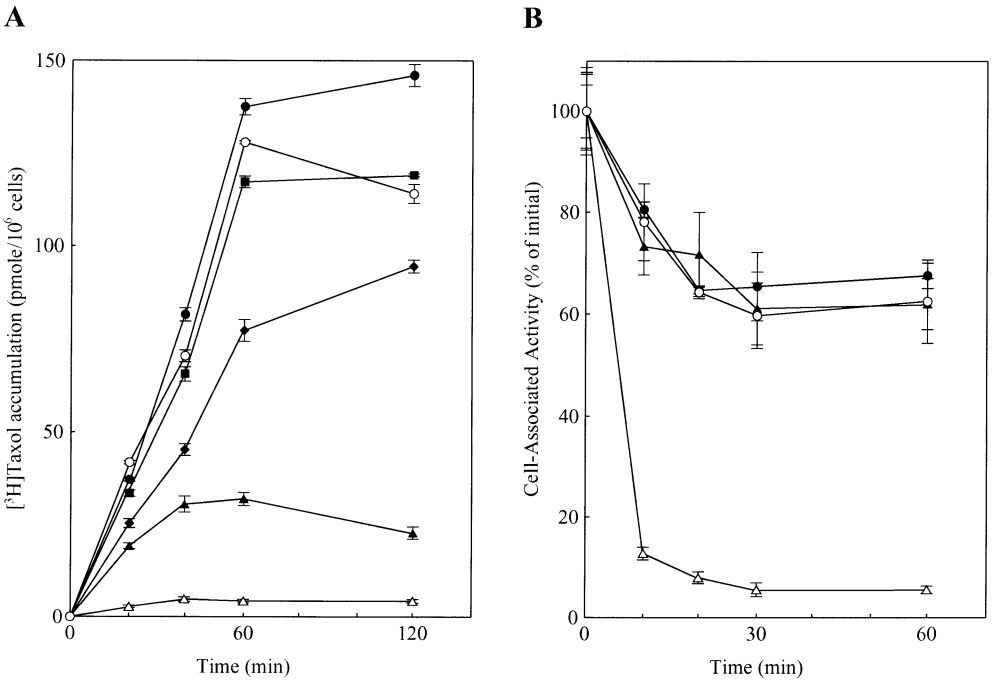

Fig. 3. Accumulation and efflux of [^{3}H]paclitaxel in KK47/WT and KK47/TX30 cells. A) [^{3}H]Paclitaxel accumulation in KK47/WT (○ and ●) and KK47/TX30 (△, ▲, ◆ and ■) cells measured in the absence (○ and △) or presence of the reversing agent N-5228 [5 μM (▲), 10 μM (◆) and 20 μM (● and ■)]. B) Efflux of [^{3}H]paclitaxel in KK47/WT and KK47/TX30 cells. [^{3}H]Paclitaxel retained in KK47/WT (○ and ●) and KK47/TX30 (△ and ▲) cells in the absence (○ and △) or presence of the reversing agent N-5228 [20 μM (● and ▲)] are shown after 1-h [^{3}H]paclitaxel accumulation. Values represent means±SEM of triplicate determinations.

paclitaxel efflux from KK47/WT cells by 1.1-fold at 30 min.

Inhibition of photolabeling by N-5228 and VLB To determine whether N-5228 could be a substrate of P-gp, we examined the inhibition of P-gp photolabeling by [^{3}H]azidopine at the indicated concentrations of N-5228 in KK47/TX30 membrane vesicles (Fig. 4). VLB was simultaneously used as a control inhibitor. As shown, N-5228 inhibited photolabeling in a dose-dependent manner. N-5228 almost completely inhibited labeling at 100 to 200 *μM*, whereas VLB inhibited labeling at 10 to 100 *μM*, suggesting that the inhibitory effect of N-5228 was slightly lower than that of VLB. However, this result demonstrated that N-5228 is a substrate of P-gp.

Structural features required to overcome paclitaxel resistance To analyze structural features, 31 new synthetic isoprenoids including N-5228 (entry 6) were examined by the MTT assay. We compared thier reversing effects at the concentration of 30 *μM*, because little effect was seen at less than 30 *μM*, except for N-5228. The results are summarized in Fig. 5 and Table III. These compounds were synthesized by modifying the structure of SDB, which is an effective P-gp modulator.[25] We prepared six different types of frameworks, which were similar to that of SDB. The structural changes were as follows: changing the aromatic ring of SDB (type I), 1,2-diamino moieties fixed with a cyclohexane ring (type II), 1,4-diamino moieties fixed with a cyclohexane ring (type III), pyrrolidine ring (type IV), benzene ring (type V), or *N*-propyl-substituted piperazine ring (type VI).

Table III shows that, of the type II compounds (entries 3 to 8) with different numbers of isoprene units, entries 3 (n=5), 4 (n=7) and 8 (n=12) exhibited strong cytotoxicity. The most suitable number of isoprene units for low cytotoxicity was 8 to 10. Type I and type IV could overcome paclitaxel resistance moderately well, but their effects were dependent on the aromatic constituents of the benzyl group. Types II and V were the most effective among the compounds analyzed, and their effects also depended on the aromatic constituents (entries 6, 11 to 22, and 26 to 29). Type VI compounds (entries 24 and 25) and N-5705 (entry 21), which had four cationic sites, were too cytotoxic to allow determination of the reversal effect. The presence of more than two cationic sites apparently increased the cytotoxicity.

We also observed that the distance between two cationic sites was an important determinant of the cytotoxicity of compounds. The distance between the two cationic sites in type III was greater than that in type II compounds. Type III compounds (entry 23), which have a fixed 1,4-diequatorial diamino moiety, exhibited stronger cytotoxicity than type II compounds (entry 6), which have a fixed 1,2-diequatorial diamino moiety on the cyclohexane ring. In addition, similar correlations were observed between type IV (entry 25) and type II compounds (entry 22). The diamino functionalities were fixed to each other at the opposite side of the C-C bond (*anti* configuration) by the pyrrolidine ring in type IV, and at the same side of the C-C bond (*syn* configuration) by the cyclohexane ring in type

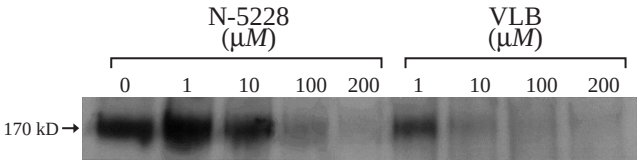

Fig. 4. Inhibition of P-gp photolabeling by [^{3}H]azidopine in KK47/TX30 membrane vesicles by N-5228 and VLB. KK47/TX30 membrane vesicles were incubated with [^{3}H]azidopine in the absence or presence of the indicated concentrations of N-5228 and VLB.

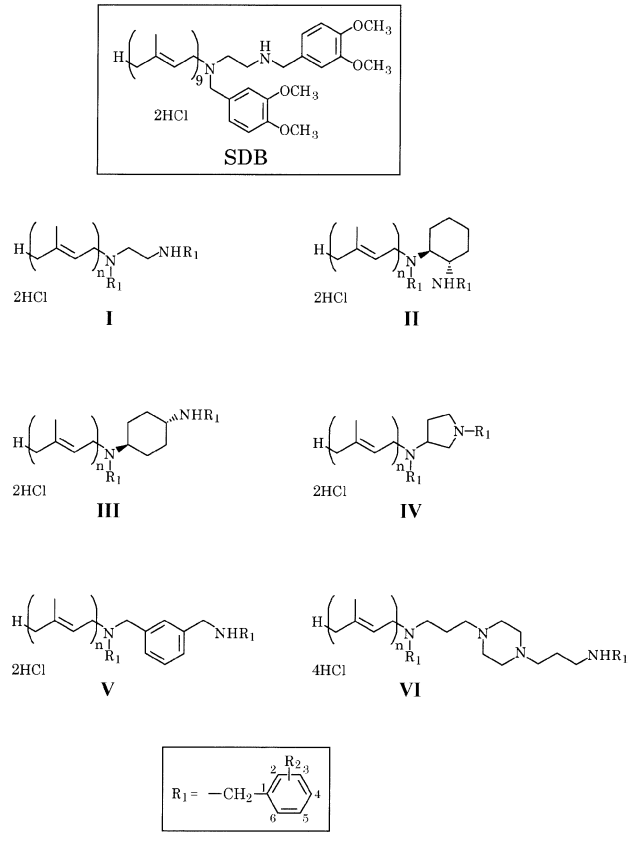

Fig. 5. Synthetic isoprenoids classified into six types. The structural features of isoprenoids were as follows: I) changing substitutions on the aromatic ring of SDB, II) 1,2-diamino moiety fixed with a cyclohexane ring, III) 1,4-diamino moiety fixed with a cyclohexane ring, IV) pyrrolidine ring, V) benzene ring, and VI) *N*-propyl-substituted piperazine ring.

Table III. Classification and Effects of Isoprenoids on Paclitaxel Resistance

Entry	Compounds	Structure			KK47/TX30		
		Type[b]	n[c]	R_1	IC_{50} (n*M*)[d]	Ratio[e]	Viability[f] (%)
	Control				4646	702	100
1	N-5230	I	9	2,3,4,5-Tetramethoxy-6-methylbenzyl	32.0	4.83	83
2	N-5229	I	9	2,4-Dimethoxybenzyl	—[g]	—	0
3	N-5707	II	5	3,4-Dimethoxybenzyl	—	—	0
4	N-5706	II	7	3,4-Dimethoxybenzyl	—	—	2
5	N-5724	II	8	3,4-Dimethoxybenzyl	6.04	0.91	65
6	N-5228	II	9	3,4-Dimethoxybenzyl	5.21	0.79	75
7	N-5690	II	10	3,4-Dimethoxybenzyl	2.15	0.32	97
8	N-5691	II	12	3,4-Dimethoxybenzyl	—	—	25
9	N-5689[a]	II	9	3,4-Dimethoxybenzyl	4.74	0.72	67
10	N-5739[a]	II	9	3,4-Dimethoxybenzyl	5.85	0.88	60
11	N-5776	II	9	4-Hydroxy-3-methoxybenzyl	6.95	1.05	65
12	N-5777	II	9	4-Methoxybenzyl	—	—	4
13	TPUMC-2	II	9	Benzyl	>300	>45.3	79
14	TPUMC-3	II	9	4-Nitrobenzyl	>300	>45.3	85
15	TPUMC-4	II	9	4-Dimethylaminobenzyl	>300	>45.3	85
16	TPUMC-5	II	9	4-Chlorobenzyl	>300	>45.3	108
17	N-5772	II	9	4-Methylbenzyl	—	—	15
18	N-5773	II	9	4-*iso*-Propylbenzyl	—	—	12
19	N-5774	II	9	4-Fluorobenzyl	—	—	24
20	N-5778	II	9	4-*n*-Butylbenzyl	—	—	2
21	N-5705	II	9	2-Pyridylmethyl	—	—	1
22	N-5779	II	9	2,3,4,5-Tetramethoxy-6-methylbenzyl	47.0	7.11	91
23	N-5740	III	9	3,4-Diethoxybenzyl	—	—	0
24	N-5741	IV	9	3,4-Diethoxybenzyl	276	41.69	54
25	N-5744	IV	9	2,3,4,5-Tetramethoxy-6-methylbenzyl	—	—	18
26	N-5257	V	9	3,4-Dimethoxybenzyl	6.06	0.91	100
27	N-5790	V	9	2,3,4,5-Tetramethoxy-6-methylbenzyl	65.9	9.96	82
28	N-5752	V	9	3,4-Difluorobenzyl	>300	>45.3	75
29	N-5753	V	9	3,4-Diethoxybenzyl	222	33.5	43
30	N-5223	VI	9	3,4-Dimethoxybenzyl	—	—	0
31	N-5742	VI	9	3,4-Diethoxybenzyl	—	—	0

a) Enantiomer.
b) Framework type in the molecule (reference to Fig. 5).
c) Number of isoprene units.
d) Cell survival was determined by MTT assay and expressed as the paclitaxel concentration that inhibited the cell growth by 50% (IC_{50}) with or without isoprenoid. Values are averages of triplicate determinations.
e) Relative resistance to KK47/WT cells without the reversing agents.
f) Cell viability was determined by MTT assay and expressed as the ratio of cellular cytotoxicity with and without isoprenoid.
g) Not evaluable because of low cell viability.

II. Therefore, the distance between two cationic sites in type IV compounds was greater than that in type II, with the result that type IV compounds exhibited stronger cytotoxicity than type II.

Lack of oxygen functionality at the C-3 position on the benzene ring in type II compounds (entries 12 to 20) decreased cell viability or was less effective at overcoming paclitaxel resistance. In type V compounds, other significant effects of aromatic constituents were observed. Both N-5790 (entry 27) with tetramethoxy functionalities (strongly electron-donating to the benzene ring) and N-5752 (entry 28) with difluoro functionalities (strongly electron-withdrawing from the benzene ring) were less effective in overcoming paclitaxel resistance than N-5257 (entry 26) with dimethoxy functionalities. Thus, the electron donation to the benzene ring was related to the rever-

sal effect, and dimethoxy functionalities provided the most suitable electron donation.

With respect to the effect of enantiomers on the reversal, no remarkable differences between the two enantiomers in type II were observed. Entry 9 (*S*,*S*-configuration) and entry 10 (*R*,*R*-configuration), which are pure enantiomers of N-5228 (entry 6), exhibited similar degrees of reversing effect to N-5228.

DISCUSSION

Paclitaxel and docetaxel have been used recently to treat a variety of cancers, including bladder cancer.[1–7] Resistance to these taxoid compounds has thus emerged as a critical issue to be resolved. We isolated a paclitaxel-resistant cell line (KK47/TX30) from the human KK47 bladder cancer cell line (KK47/WT) in order to investigate the mechanism of, and reversal agents for, paclitaxel resistance. KK47/TX30 cells exhibited approximately 700-fold resistance to paclitaxel compared with the parental cells, and showed cross-resistance to VLB, VCR, ADM, VP-16 and MITO. This cross-resistance pattern is similar to that reported in many P-gp-mediated MDR cell lines.[10–12]

Paclitaxel stabilizes microtubules consisting of both α- and β-tubulin by disrupting the dynamic equilibrium between soluble tubulin dimers and their polymerized form.[40] Previous studies reported that tubulin polymerization decreased in paclitaxel-resistant cell lines in paclitaxel-containing hypotonic buffer.[29, 41] We compared the ratio of polymerized α- and β-tubulin between the parental KK47/WT and the resistant KK47/TX30 cells. However, there was no significant difference in the ratio of polymerized tubulin between them. This result suggests that there was no major alteration in tubulin function in our cells.

Western blot analysis demonstrated that P-gp and LRP were overexpressed in KK47/TX30 cells. LRP is the major vault protein associated with nucleocytoplasmic transport.[17–20] A previous study demonstrated that LRP overexpression is associated with paclitaxel resistance, and LRP may be involved in nucleocytoplasmic transport of paclitaxel.[21] However, in the present study, it is unlikely that LRP played a major role in the acquisition of paclitaxel resistance, as LRP was only weakly expressed in resistant cells compared with P-gp in the same volume of membrane vesicles. In addition, conventional P-gp-mediated MDR-reversing agents, such as verapamil and cepharanthine, could completely overcome paclitaxel resistance in KK47/TX30 cells.

Previous studies reported that the mechanisms of taxoid resistance include P-gp overexpression,[8] and molecular alterations of microtubule genes and tumor suppressor genes, including *p53*.[9] Consistent with previous reports,[10–12] paclitaxel resistance in our model cells appeared to be associated with P-gp overexpression. Our present study demonstrated that paclitaxel accumulation in the resistant cells decreased to 1/25 of that in the parental cells after 60 min of incubation, that decreased paclitaxel accumulation in the resistant cells was due to enhanced paclitaxel efflux, and that N-5228, a substrate of P-gp, completely reversed paclitaxel resistance in the resistant cells. These findings strongly suggest that paclitaxel resistance in our cells was primarily mediated by P-gp.

In the present study, we investigated whether new synthetic isoprenoids could overcome paclitaxel resistance in this cell line. To this end, we synthesized 31 isoprenoids by modifying SDB. Among them, we found that N-5228 could completely overcome paclitaxel resistance at a lower concentration (20 μM) than *N*-(*p*-methylbenzyl)decaprenylamine (PMB) and SDB [PMB at 216 μg/ml (258 μM) and SDB at 34 μg/ml (35 μM)], which we previously reported as P-gp-mediated MDR-reversing agents.[23] A photoaffinity labeling study revealed that N-5228 inhibited P-gp photolabeling by [^{3}H]azidopine in a dose-dependent manner, suggesting that N-5228 could bind to P-gp directly and could be a substrate of P-gp. In comparison with the concentration required to inhibit photolabeling, N-5228 (100 to 200 μM) was a slightly weaker inhibitor than vinblastine (10 to 100 μM), and than verapamil (10 to 100 μM), as was demonstrated by Zordan-Nudo *et al.*[42] Taken together, these findings indicate that it is necessary to develop compounds that work effectively at lower concentrations.

We therefore investigated structural features of the isoprenoids that could reverse paclitaxel resistance in order to identify the structural requirements for the reversal and to facilitate development of more potent reversal agents. Zhang *et al.*[43] previously investigated structural features of P-gp modulators, including prenylcystine methyl esters, by measuring the ATPase activity of P-gp. They found that the required structural elements of isoprenoids for interaction with P-gp included the isoprenoid moiety, the positive charge of the protonated amino group, and the carboxyl methyl group. Wiese and Pajeva[44] reviewed the common denominators of essential structures for P-gp modulators, and concluded that they included, 1) hydrophobic moieties, 2) cationic sites, and 3) methyl moieties. Seelig *et al.*[45] analyzed electron donor patterns among 100 P-gp substrates including SDB. They concluded that the binding to P-gp increased with the strength and the number of electron donor groups. This is consistent with our photoaffinity labeling study results, because VLB has more electron donor groups than N-5228. However, our study demonstrated that “di-”methoxy functionalities were more effective than “tetra-”methoxy functionalities. This result suggests that the binding affinity to P-gp is not always associated with the degree of reversing effect. Further study is necessary to elucidate the structure-activity relationships.

In conclusion, we isolated a paclitaxel-resistant cell line (KK47/TX30) overexpressing P-gp from a human bladder cancer cell line. KK47/TX30 cells exhibited decreased paclitaxel accumulation due to increased paclitaxel efflux. We observed that one of 31 new synthetic isoprenoids, N-5228, could completely overcome P-gp-mediated paclitaxel resistance. We investigated the structural features of these 31 isoprenoids. Our results suggest that the following structural features are important for overcoming paclitaxel resistance: 1) a basic structure of 8 to 10 isoprene units, 2) a cyclohexane or benzene ring within the framework, 3) two cationic sites in close proximity to each other, and 4) a benzyl group having 3,4-dimethoxy functionalities, which provide moderate electron-donation. It appears that these structural features are necessary to modulate P-gp function in paclitaxel-resistant cells. However, more precise studies with more compounds are needed to elucidate the correlation between structure and activity.

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