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Preferential Binding of E7010 to Murine β3-Tubulin and Decreased β3-Tubulin in E7010-resistant Cell Lines

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N-[2-[(4-Hydroxyphenyl)amino]-3-pyridyl]-4-methoxybenzenesulfonamide (E7010) is a novel sulfonamide antimitotic agent, which is active against mouse and human tumors. E7010 binds to β-tubulin and inhibits polymerization of microtubules. In order to clarify the mechanisms of E7010-resistance, two murine leukemic P388 subclones resistant to E7010, 0.5r-D and 4.0r-M, were characterized. The two clones showed approximately 10- and 100-fold resistance to E7010-induced growth-inhibitory effects, respectively, compared with the parental cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. These cell lines showed no cross-resistance to other anticancer agents such as taxanes, vinca alkaloids, mitomycin C, cisplatin and irinotecan hydrochloride (CPT-11). Increased α- and β-tubulin protein and mRNA levels were observed in 0.5r-D and 4.0r-M cells as compared with the parental cells. We examined the isotype-specific expression of β-tubulin in these E7010-resistant cells by a competitive reverse transcription-polymerase chain reaction method. Although a 50% increase in β5 isotype mRNA levels was observed in 4.0r-M cells, the levels of β3 isotype message in the two resistant clones were approximately 50% less than the parental cells. To elucidate the binding properties of E7010 with β-tubulin isotypes, we prepared isotype-specific fusion proteins of β-tubulins. Direct photoaffinity labeling of the isotype-specific fusion proteins with [^{14}C]E7010 revealed that E7010 preferentially binds to the β3 isotype rather than β2, β4, and β5 isotype proteins. Therefore, altered expression of β-tubulin isotypes, especially β3 isotype, to which E7010 binds with high affinity, may account for the decreased sensitivity of these resistant clones to E7010.

Key words: E7010 — Antimicrotubule agent — Drug resistance — β3-Tubulin

N-[2-[(4-Hydroxyphenyl)amino]-3-pyridyl]-4-methoxybenzenesulfonamide (E7010) is a novel sulfonamide antimitotic agent which binds to tubulin and inhibits the assembly of microtubules.[1)] E7010 shows a wide therapeutic range of antitumor activity in the colon 38 carcimoma model.[2)] It is also active against mouse and human tumors.[2)] In a phase I study of E7010 using a single-dose schedule, some responses were observed, and a phase I study of 5-day continuous administration is in progress. An *in vitro* study showed that E7010 arrests the cell cycle in the G2/M phase, and that it binds to the same site as colchicine.[1)]

The α- and β-tubulin proteins are encoded by separate genes.[3,4)] The β-tubulin gene consists of 6 isotypes which are highly conserved across vertebrate species and differ from each other predominantly at the carboxy terminus, where the microtubule-associated protein binding sites are located. Differences in the levels of expression of the β-tubulin isotypes were reported in cell lines resistant to antimicrotubule agents such as paclitaxel and estramustine.[5,6)] Therefore, differences in the expression of the β-tubulin isotypes may affect sensitivity to antimicrotubule agents. Recently Banerjee *et al*. demonstrated that changes in the relative amounts of the different tubulin isotypes alter microtubule dynamics.[7)] However, the functional difference between the tubulin isotypes remains unclear. In the present study, the relative levels of expression of β-tubulin isotypes in E7010-resistant murine leukemic cells were determined by competitive reverse transcription-polymerase chain reaction, to examine whether different levels of expression of these isotypes contribute to the resistance of these cells to antimitotic agents. Furthermore, we demonstrated that E7010 exhibited isotype-specific binding by direct photoaffinity labeling of the isotype-specific tubulin fusion proteins. This approach using isotype-specific proteins of β-tubulin is considered to be a useful means to determine the potency and properties of antimitotic drugs.

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MATERIALS AND METHODS

Chemicals E7010 and [^{14}C]E7010 (specific activity, 56 mCi/mmol; phenyl-U-RING-^{14}C) were provided by Eisai Co., Ltd. (Tsukuba). Vindesine and vincristine were purchased from Shionogi Co., Ltd. (Osaka). Paclitaxel, cisplatin and etoposide were obtained from Bristol-Myers Squibb Co., Ltd. (Tokyo). RPMI 1640 medium and phosphate-buffered saline (PBS) were purchased from Nissui Co., Ltd. (Tokyo).

Cell lines P388 murine leukemia was supplied by Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo). Two E7010-resistant cell lines, P388/0.5r-D and P388/4.0r-M, were established at Tsukuba Research Laboratories, Eisai Co., Ltd.[1)]

Growth inhibition assay To determine the growth-inhibitory effect of drugs, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[8)] A well containing only RPMI 1640 medium, 10% fetal bovine serum and MTT (Sigma, St Louis, MO) was used as a control. Each experiment was performed in 6 wells for each concentration required for a 50% reduction of the optical density in each test, and was calculated as: (mean absorbance in six wells containing drug−absorbance in six control wells)/(mean absorbance in six drug-free wells −absorbance in six control wells)×100. The relative resistance was defined as the IC_{50} of the resistant subline/IC_{50} of the parental cell line.

Northern blot analysis RNA was extracted from the parent and E7010-resistant cell lines by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[9)] Twenty micrograms of total RNA was electrophoresed on a 1% agarose-6% formaldehyde gel at 25 V for 12 h. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham, Tokyo), which was then hybridized overnight with an [α-^{32}P]dCTP-labeled DNA probe at 42°C. The α- and β-tubulin probes were prepared by polymerase chain reaction (PCR), subcloned into PCR2 (Invitrogen, San Diego, CA), sequenced by an ABI PRISM 310 genetic analyzer (Perkin Elmer, Foster City, CA) and then linearized by *Eco*RI digestion. The sequences of the primers were as follows (Nippon Seihun, Kanagawa): α-tubulin sense, 5′-GAATTCCAGACCAACCTGGT-3′; antisense, 5′-GTGTTGCTCAGCATGCACAC-3′; β-tubulin sense, 5′-CACTTCTTCATGCCTGGCTTT-3′; antisense, 5′-CTGGTAC-TCAGACACCAGGTC-3′. The probe was labeled with [α-^{32}P]dCTP by means of a multiprime labeling system kit (Amersham). After hybridization, the membrane was washed three times with 2× SSC (1× SSC consists of 0.15 *M* NaCl and 0.015 *M* sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 10 min. After 3 washes with 0.1× SSC and 0.1% SDS for 15 min at 65°C, followed by several washes with 0.1× SSC, the membrane was exposed to an imaging plate (BAS-III, Fuji Film, Tokyo). The plate was analyzed by a BAS 2000 Bio-image analyzer (Fuji Film).

Preparation of cell lysates for determination of total and polymerized tubulin content Total tubulin was isolated by a modification of the method reported by Thrower *et al.*[10)] and Minotti *et al.*[11)] Exponentially growing cells were washed twice with PBS, collected and adjusted to a concentration of 1×10^6 cells/ml. One milliliter of each cell suspension was centrifuged at 200*g* for 5 min, and resuspended in 0.3 ml of depolymerization buffer (0.1 *M* 2-[N-morpholino]ethanesulfonic acid, 1 m*M* $MgSO_4$, 10 m*M* $CaCl_2$, and 5 m*M* guanine triphosphate, pH 6.9). The cells were lysed by sonication on ice with a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) at 15 W output for two intervals of 15 s each. Lysate fractions were incubated in depolymerization buffer for 1 h on ice to depolymerize the microtubules, then the samples were centrifuged at 50,000*g* for 15 min at 4°C using a TL-100 centrifuge (Beckman, Irvine, CA) with a fixed-angle TL-45 rotor (Beckman). The supernatant was transferred to a new centrifuge tube. After addition of 30 μl of protein G Sepharose 4 Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden) to samples and mixing for 1 h at 4°C, the tube was centrifuged at 250*g* for 5 min. The supernatant was transferred to a new centrifuge tube, anti-α or anti-β antibodies were added and the tube was mixed for 1 h at 4°C. Thirty microliters of protein G Sepharose was then added to each sample, following by mixing for 1 h at 4°C and washing 3 times with TNE buffer (10 m*M* Tris-HCl, pH 7.6, 1%(w/v) Nonidet P-40 (Iwai Chem. Co., Tokyo), 0.15 *M* NaCl, and 1 m*M* ethylenediaminetetraacetic acid (EDTA)).

Polymerized tubulin was isolated by a modification of the method of Thrower *et al.*[10)] Briefly, exponentially growing cells were washed twice with warmed PBS, collected and adjusted to 4×10^6 cells/ml. One milliliter of each cell suspension was centrifuged at 200*g* for 5 min. The pellet was resuspended in 1 ml of stabilization buffer (20 m*M* Tris-HCl, pH 6.8, 0.14 *M* NaCl, 0.5% Nonidet P-40, 1 m*M* $MgCl_2$, 2 m*M* ethyleneglycol-bis(aminoethylether)-N,N,N′,N′-tetraacetic acid, 4 μg/ml paclitaxel) and incubated at 37°C for 30 min. Each sample was centrifuged at 50,000*g* for 15 min at 37°C. The supernatant was aspirated and the pellet was resuspended in 0.3 ml of depolymerization buffer. The lysates were incubated on ice for 1 h, following by centrifugation and immunoprecipitation in the same manner as described for total tubulin isolation.

Western blot analysis of total and polymerized tubulin P388 cells and E7010-resistant cells were exposed to 160 n*M* E7010 for 24 h, and total and polymerized tubulin were prepared as described above. The contents of total tubulin were analyzed on a 10% SDS-polyacrylamide gel. After electrophoresis, protein was transferred electro-

phoretically to a nitrocellulose membrane as reported by Towbin *et al.* [12] The membrane was incubated with blocking buffer (5% skim milk with PBS) for 1 h and then allowed to react with anti-α- or anti-β-tubulin antibodies for 12 h at 4°C. After incubation, the membrane was washed four times with PBS containing 0.1% Tween-20 and incubated with biotinylated anti-mouse IgG antibody at room temperature for 1 h. The bands were detected by using a Peroxidase Immunostain set (Wako Pure Chemical Industries, Tokyo), and analyzed with an Ultrascan XL enhanced laser densitometer (Pharmacia LKB).

Analyses of β-tubulin mRNA expression by competitive reverse transcriptase-PCR (RT-PCR) Four oligonucleotides (named A, T7/A, B, BC) were used to generate each cRNA construction. All oligonucleotides listed in Table IA were synthesized and purified by Nippon Seihun. Each T7/A primer corresponds to primer A oligonucleotides carrying a 37 base extension at the 5′ end (5′-CGGGATCCGGATCCTAATACGACTCACTATA GGGAGA-3′) containing the T7 RNA polymerase promoter region. Primers BC have 20 nucleotides at the 5′ end (B) that correspond to the target sequence *n* bases upstream from (C). These β-tubulin isotype sequences for murine β1, β2, β3, β4, and β5 were derived from the published sequences of these genes. The unpublished nucleotide sequence of β6 was provided by Drs. N. Cowan and S. Lewis (Dept. of Biochemistry, New York Univ. School of Medicine, New York, NY). The antisense primers were selected from sequences corresponding to the 3′-untranslated regions.

Competitor RNA was synthesized by the recombinant PCR procedure. PCR was initially performed using the primers A and B. The second round of PCR was performed using T7/A and BC primers. PCR products were then purified from an agarose gel using a Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). They were transcribed using T7 RNA polymerase-based *in vitro* transcription kit (Nippon Gene, Tokyo). The remaining DNA templates were digested twice with DNase. Finally, the synthetic competitor RNAs were purified by chromatography on Chroma Spin-100 diethyl-pyrocarbonate-treated H_2O columns (Clontech, Palo Alto, CA), quantified by measuring their absorbance at 260 nm, diluted in TE (10 m*M* Tris-HCl, 1 m*M* EDTA) buffer and stored at −80°C until use.

A constant amount of each RNA sample was added to increasing known amounts of cRNA, then the RNA mixtures were reverse-transcribed with primer D. The RT products were subjected to PCR amplification with [α-^{32}P]dCTP. Following an initial denaturation at 97°C for 4 min, 35 cycles at 97°C for 45 s, 57°C for 1 min, and 72°C for 2 min were performed. The PCR products were separated on a 5% polyacrylamide gel, which was then dried. The ratios of the intensities between the competitor and target PCR products were plotted against the concentration of the competitor RNA samples. To determine the

Table I. Origonucleotide Primers Used for Amplification and Sequencing of β-Tubulin Isotypes (A) and Isotype-specific Fusion Proteins (B)

		Site of primer	Size of construction	
			Competitor	Target
A. Oligonucleotide primers for competitive RT-PCR				
β1	A: 5′-GGA GGT GGA TGA GCA GAT GC-3′	1009		
	B: 5′-GAC AGA GGC AAA CTG AGC A-3′	1386	417	465
	C: 5′-CTA AGG GTG CAC ACT GTA TC-3′	1454		
β3	A: 5′-GCC TGT GAT CCA AGA CAT-3′	941		
	B: 5′-GGC TGT GAA TGA ATA AAG-3′	1310	405	492
	C: 5′-AGC TGT AGC GTC CTG GTA-3′	1415		
β5	A: 5′-CCA GCC GTG GAA GCC AGC AG-3′	899		
	B: 5′-GTG CCA GGC ACC ATT TAC CC-3′	1434	575	661
	C: 5′-GGG ATC TAA GCA GCC TGA GC-3′	1540		
B. Oligonucleotide primers for fusion proteins				
β2	up: 5′-GTG GAA TTC TAT AAT GCC ACC CTC TCA GTG-3′			
	down: 5′-ATT GTC GAC TAG AAA GAC CAA TGC TGG A-3′			
β3	up: 5′-CTT GAA TTC ATC TGC TTC AGA ACC CTA AA-3′			
	down: 5′-ACA GTC GAC AGC TAA TGC ACA GTG GAC A-3′			
β4	up: 5′-CAT GAA TTC GTG GAG AAC ACT GAT GAG ACC-3′			
	down: 5′-CAA TCT AGA GCA GGG GTG TAT GCT TAG G-3′			
β5	up: 5′-CCC GAA TTC GCC ACC CTG TCT GTC CAT CA-3′			
	down: 5′-GAA GTC GAC AGG GAT CTA AGC AGC CTG A-3′			

expression of specific isotypes of β-tubulin, we used a competitor/target ratio of 1.

Synthesis of fusion proteins of specific β-tubulin isotypes The β2, β3, β4 and β5-isotype genes were amplified from P388 cells by the RT-PCR method using upper and lower primers which had additional restriction enzyme sites for *Eco*RI and *Sal*I (β2, β3, β5), or *Eco*RI and *Xba*I (β4) (Table IB). After having been subcloned into the PCRII plasmid vector, PCR products were sequenced on an ABI PRISM 310 genetic analyzer. After cloning of each insert into the pMAL-c2 vector (New England Biolabs, Beverly, MA), *Escherichia coli* JM109 host cells were transfected with these clones and heat-shocked to induce expression of maltose binding protein isotype fusion proteins. Fusion proteins were isolated by affinity chromatography and then concentrated using a Centricon-10 centrifuge (Amicon). The proteins were confirmed by western blot analysis using two different anti-β-tubulin monoclonal antibodies, monoclonal anti-β-tubulin antibody (Sigma) and sea urchin eggs (mouse) monoclonal antibody (Calbiochem, La Jolla, CA). Each fusion protein was stored at −20°C until use.

Photoaffinity labeling of crude extracts and immunoprecipitated tubulin of P388 cells and E7010-resistant cell lines Total tubulin and immunoprecipitated tubulin of the three cell lines were isolated as described above. Aliquots of 40 μg protein and immunoprecipitate (from 1×10^6 cells/ml) sample were incubated with 10 m*M* [^{14}C]E7010 at 37°C for 30 min. Samples were then irradiated at 254 nm and 365 nm for 30 min, respectively, at a distance of 7 cm on ice. After irradiation, the samples were applied to a 12% SDS-polyacrylamide gel. The gel was dried and exposed to an imaging plate, which was analyzed on a BAS 2000 Bio-image analyzer. Normal tubulin, purified tubulin and MAP-rich tubulin, which were obtained from Cytoskeleton (Santa Barbara, CA), were labeled with [^{14}C]E7010 in the same way as controls.

Photoaffinity labeling of β-tubulin isotype-specific fusion proteins Two micrograms of each fusion protein was incubated with 10 m*M* [^{14}C]E7010 and unlabeled 500 m*M* E7010 or 500 m*M* colchicine at 37°C for 30 min. Samples were then irradiated at 254 nm and 365 nm for 30 min, respectively, at a distance of 7 cm on ice. After irradiation, the samples were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The gel was dried and exposed to an imaging plate. The plate was analyzed on a BAS 2000 Bio-image analyzer.

RESULTS

Sensitivities of the E7010-resistant cell lines to various anticancer agents The growth-inhibitory effects of E7010 and other anticancer agents in P388 parental, P388/0.5r-D, and P388/4.0r-M cells were evaluated by MTT assay, and the IC_{50} values are shown in Table II. The relative resistances of P388/0.5r-D and P388/4.0r-M cells to E7010 were 8.8- and 112.5-fold, respectively. The two E7010-resistant clones showed no cross-resistance to other anticancer agents such as taxanes, vinca alkaloids, mitomycin C, cisplatin or CPT-11. These results are consistent with a previous report by Yoshimatsu *et al.*,[1] which demonstrated that none of the E7010-resistant cell lines showed cross-resistance to any of the antitumor drugs tested which target sites other than colchicine-bind-

Table II. Growth-inhibitory Effect (IC_{50})[a] of Various Anti-cancer Agents on P388 and E7010-resistant Cells[b]

Drug	P388	P388/0.5r-D	RR[c]	P388/4.0r-M	RR[c]
E7010 (μM)	0.16±0.03	1.4±0.1	8.8	18±0.68	112.5
Vindesine (n*M*)	5.0±2.0	7.7±1.5	1.5	5.5±1.3	1.1
Vincristine (n*M*)	3.5±3.5	5.0±1.4	1.4	2.8±2.5	0.8
Taxol (n*M*)	14±5.5	7.4±3.1	0.5	14±4.8	1.0
Taxotere (n*M*)	21±6.4	8.8±3.0	0.4	25±13	1.2
Adriamycin (n*M*)	62±10	75±35	1.2	83±29	1.3
Mitomycin C (n*M*)	84±30	81±30	1	130±45	1.5
Cisplatin (n*M*)	320±260	230±76	0.7	180	0.6
CPT-11 (n*M*)	13±7.2	14±6.9	1.1	15±5.1	1.1
Etoposide (n*M*)	19±1.7	27±1.7	1.4	29±1.7	1.5

a) The 50% inhibitory concentration (IC_{50}) values were obtained from dose-response curves with 8 different drug concentrations.
b) Each cell line was continuously exposed to each drug for 72 h. The growth-inhibitory effect of the drugs was measured by MTT assay. Values are means±SD of 3 independent experiments.
c) RR, relative resistance value equals the IC_{50} value of the resistant cell lines divided by the IC_{50} value of the parent cell line.

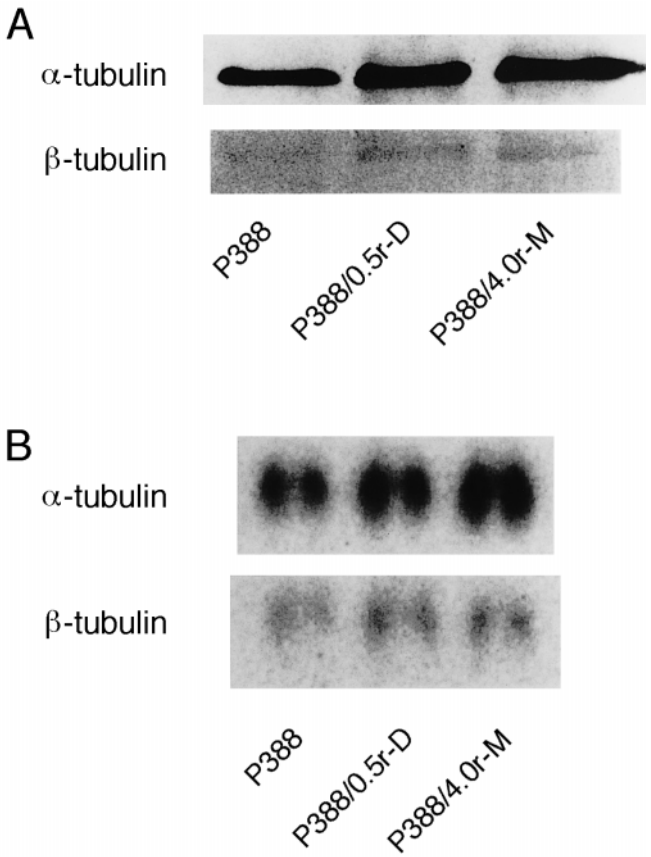

Fig. 1. Total tubulin content in P388 and E7010-resistant cells. A. Western blot analysis of α- and β-tubulin in P388 and E7010-resistant cell lines with α- and β-tubulin antibodies. A total of 10 μg of protein was loaded onto each lane. B. Northern blot analysis of α- and β-tubulin in P388 and E7010-resistant cell lines. Total RNA (10 μg) from each cell line was electrophoretically separated and transferred to nylon membranes. Blots were hybridized with 360-base-pair oligonucleotides (α-tubulin) and 450-base-pair oligonucleotides (β-tubulin) as probes.

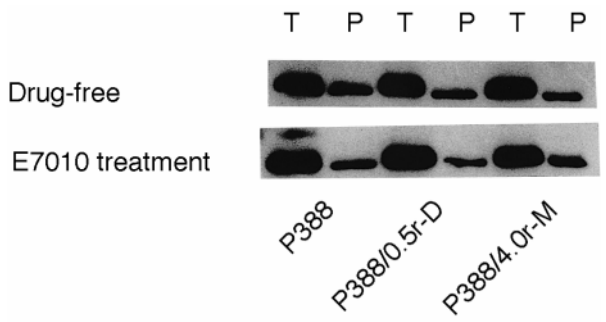

Fig. 2. Western blot analysis of β-tubulin in P388 and E7010-resistant cells after exposure to E7010 (160 n*M*) for 24 h. T, total tubulin; P, polymerized tubulin.

ing sites. To confirm that P388/0.5r-D and P388/4.0r-M clones did not show a Pgp-mediated multidrug-resistance phenotype, we compared P-glycoprotein expression in P388/0.5r-D and P388/4.0r-M cells by flow cytometry using MRK16, an anti-Pgp mouse IgG Mab provided by Prof. T. Tsuruo (Tokyo University, Tokyo), and found no difference in expression between P388/0.5r-D, P388/4.0r-M and P388 cells (data not shown). Based on these results, we speculate that the mechanisms of resistance might involve some alteration of tubulin in these E7010-resistant cell lines.

Tubulin content in E7010-resistant cells In order to elucidate the mechanism of resistance in the E7010-resistant cells, we first compared the protein contents of α- and β-tubulin in these cells. The two E7010-resistant cell lines showed increased levels of both α-tubulin (×1.5) and β-tubulin (×1.5) as compared with the parental cell line (Fig. 1A). To determine whether the overall increase in α- and β-tubulin occurred at the transcriptional level, α- and β-tubulin mRNA levels were determined in these cells by northern blot analysis with α- and β-tubulin probes. The P388/0.5r-D and P388/4.0r-M cells showed 1.7-fold and 2.2-fold increases in β-tubulin mRNA levels, and a 1.3-fold increase in β-tubulin protein (Fig. 1B). These results suggest that the increase in tubulin levels in these E7010-resistant cells was mediated at the transcriptional level. However, the degree of increase in tubulin levels was independent of the level of E7010 resistance in each of the cell lines. Since a decreased polymerized tubulin content is considered to be one property of cells that are resistant to antimitotic agents, the ratio of polymerized tubulin/total tubulin was examined, but there was no difference in the polymerized tubulin ratio among the P388, P388/0.5r-D, and P388/4.0r-M cell lines under drug-free conditions (Fig. 2). Although the polymerized tubulin content decreased in the parental cell line following exposure to 160 n*M* E7010 for 24 h, there was no change in P388/4.0r-M cells. The total tubulin contents did not change after drug exposure in the three cell lines.

Analysis of β-tubulin isotype mRNA expression by competitive RT-PCR To clarify the relationship between the differential expression of β-tubulin isotypes in P388, P388/0.5r-D, and P388/4.0r-M, we performed isotype-specific competitive RT-PCR. The high levels of sequence conservation within the coding regions of β-tubulin genes suggested that isotype-specific oligonucleotide primers should be designed within the nonconserved untranslated regions (UTR) of these genes. We designed the downstream primers at the 3′-UTR. Synthetic competitor RNAs (cRNAs) allowing the quantification of each β-tubulin isotype was generated by *in vitro* transcription of modified β2, β3, β4 and β5 PCR products. We could not detect the β1 and β6 isotypes by RT-PCR. PCR using a β4-specific primer produced a faint band from mRNA of all

three cell lines, but this disappeared following addition of a competitor. Deletions were created by the recombinant PCR procedure, as schematically illustrated in Fig. 3A. The β2 isotype appeared to be expressed at equivalent levels in all cell lines. A 50% decrease in β3 isotype message levels was observed in P388/0.5r-D and P388/4.0r-M cells (Fig. 3B) and a 2-fold increase in β5 isotype mRNA was observed in P388/4.0r-M cells (Fig. 3B).

Photoaffinity labeling of crude tubulin extracts and immunoprecipitated tubulin of P388 cells and E7010-resistant cell lines There were no obvious differences of E7010-binding to tubulin in the crude extracts and immunoprecipitates between the three cell lines (Fig. 4, A and B) On the other hand, binding of E7010 to the total tubulin seemed to be increased in the resistant clones. It appeared that the binding capacity of total tubulin for E7010 was increased in the resistant cells.

Direct photoaffinity labeling of fusion proteins with E7010 To examine the binding of E7010 to tubulin, we prepared specific fusion proteins of murine tubulin β2, β3, β4, and β5 isotypes (Fig. 5A) and examined the photoincorporation of [^{14}C]E7010 into these proteins.

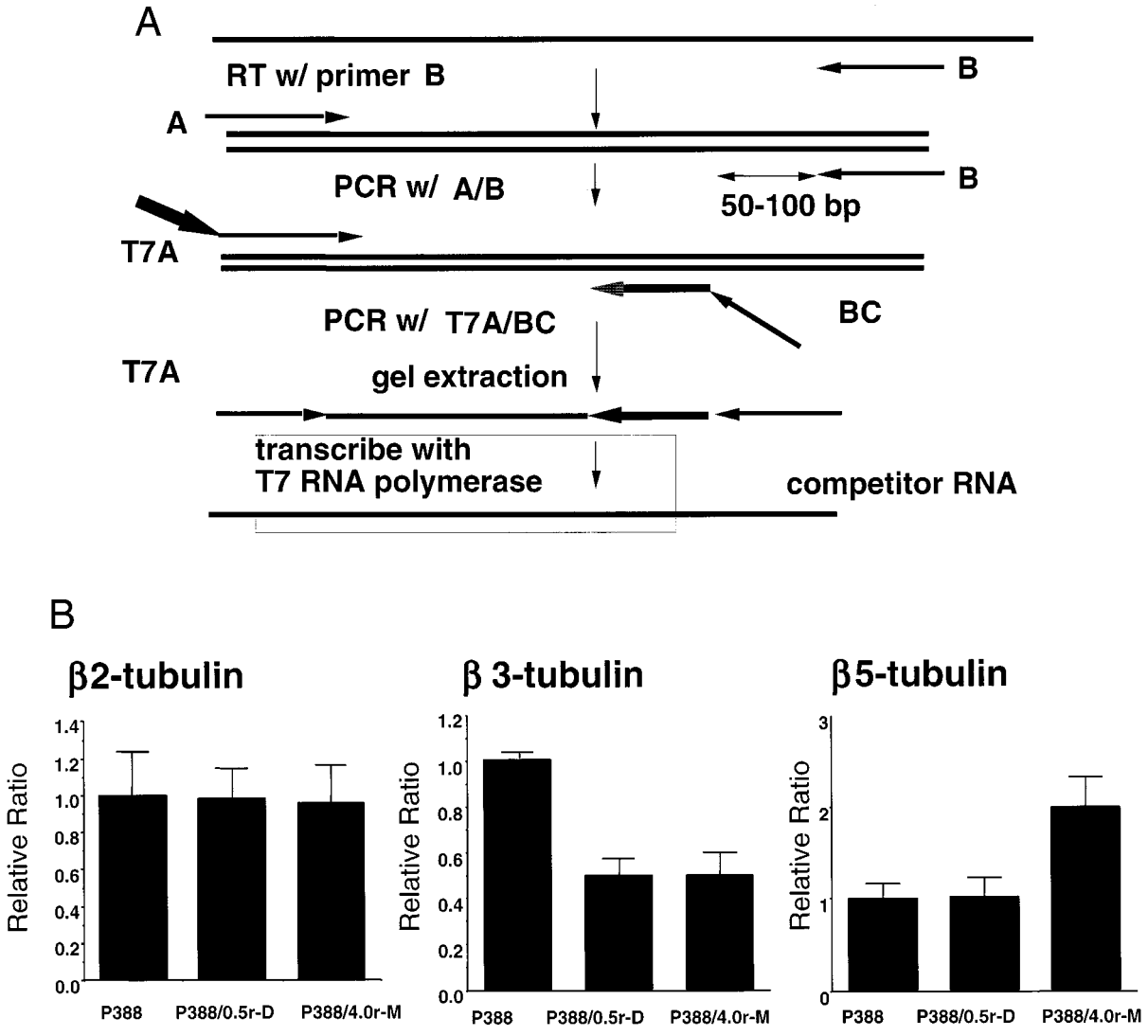

Fig. 3. Quantitative analysis of mRNA of β-tubulin isotypes by competitive RT-PCR. A. Construction of competitor RNAs (for details, see "Materials and Methods"). B. mRNA expressions of β-tubulin isotypes evaluated by competitive RT-PCR. The vertical axis represents the relative ratio based on expression of the parental cells' mRNA as 1. These values were determined in at least three experiments. Columns, mean; bars, β SD; $P<0.05$. Ratio, (competitor/target).

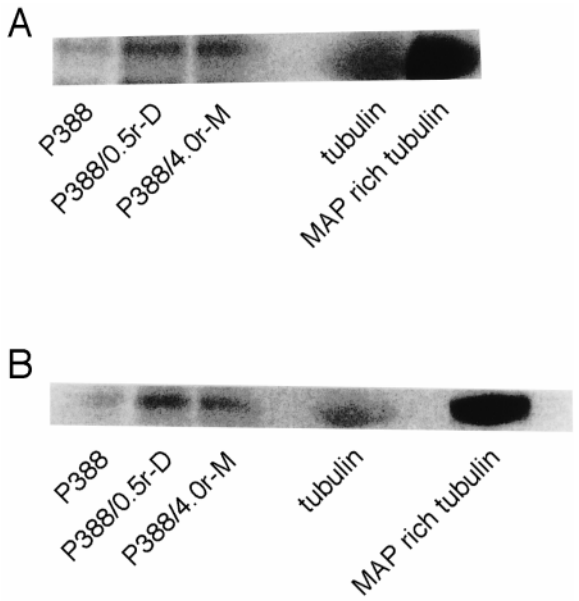

Fig. 4. Photoaffinity labeling of crude extract of P388 and E7010-resistant cells with [^{14}C]E7010. A. Photoincorporation of [^{14}C]E7010 into crude extract of P388 and E7010-resistant cells. Each crude extract (40 μg protein) was incubated with an equal amount of [^{14}C]E7010, cross linked by ultraviolet irradiation and electrophoresed. B. Photoincorporation of [^{14}C]E7010 into β-tubulin-immunoprecipitates of P388 and E7010-resistant cells. Each immunoprecipitate (from 1×10^6 cells/ml) was incubated with an equal amount of [^{14}C]E7010, cross linked by ultraviolet irradiation and electrophoresed.

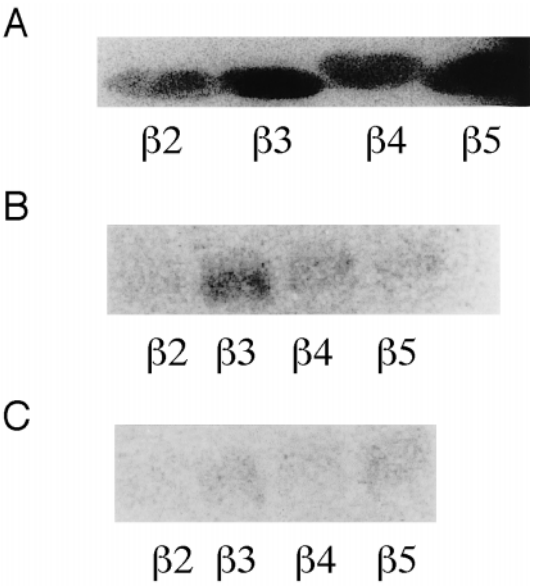

Fig. 5. Photoaffinity labeling of isotype-specific β-tubulin proteins fused to myelin basic protein with [^{14}C]E7010. The fusion proteins were prepared and labeled as described in "Materials and Methods." A. Western blot analysis of β-tubulin isotype-specific fusion proteins. B. Photoaffinity labeling of β-tubulin isotype-specific fusion proteins with [^{14}C]E7010. Each fusion protein (2 μg) was incubated with an equal amount of [^{14}C]E7010 and a 50-fold excess of cold E7010, cross linked by ultraviolet irradiation and electrophoresed. C. Photoaffinity labeling of β-tubulin isotype-specific fusion proteins with [^{14}C]E7010 in the presence of colchicine. Each fusion protein (2 μg) was incubated with an equal amount of [^{14}C]E7010 and a 50-fold excess of cold colchicine, cross linked by ultraviolet irradiation and electrophoresed.

Although the photoincorporation of [^{14}C]E7010 into the β2, β4 and β5 tubulins was reduced by addition of a 50-fold excess of cold E7010, that into the β3 isotype was not reduced by addition of cold E7010 (Fig. 5B). All the fusion proteins showed reduced photoincorporation following addition of a 50-fold excess of cold colchicine (Fig. 5C). This result suggests that E7010 binds to the same sites as colchicine, and that E7010 preferentially binds to the colchicine binding site of the β3 isotype.

DISCUSSION

Various antimitotic agents are used for cancer chemotherapy. They mainly bind to β-tubulin and affect the polymerization and depolymerization of microtubules. E7010 is a unique sulfonamide antimitotic agent that binds to β-tubulin, probably at the same binding site as colchicine.[1)] It inhibits tubulin polymerization and arrests the cell cycle at G2/M.[1)] We demonstrate here that E7010 preferentially binds to the colchicine binding sites of β3 tubulin.

The MTT assay revealed that several other types of anticancer drug-resistant cell lines, including P-glycoprotein-mediated multidrug resistant phenotypes, show no cross-resistance to E7010. In addition, intracellular accumulations of [^{14}C]E7010 were similar in the parental and E7010-resistant cell lines (data not shown). Therefore, we speculate that tubulin changes in the cells may contribute to the resistance phenotype, since E7010 is a tubulin-binding agent. Total α- and β-tubulin contents were increased in the E7010-resistant cell lines at the protein and mRNA levels. There was no statistically significant difference in E7010 binding to the cellular tubulin between P388 and E7010-resistant cells, but binding of E7010 to the total tubulin seemed to be increased in the resistant clones (Fig. 4, A and B). Because increased binding of the drug to target molecules usually causes increased sensitivity, it is difficult to explain the resistance mechanism in terms of increased drug binding of total tubulin. Since E7010

inhibits tubulin polymerization, we compared the relative ratios of polymerized tubulins in parental and resistant cell lines before and after exposure to E7010 (Fig. 2). There was no marked difference in polymerization ratio between the cell lines under non-treatment conditions. In parental cells and P388/0.5r-D cells, the polymerized tubulin content was decreased after exposure to E7010. In contrast, in the presence of E7010, the ratio of polymerized tubulin to total tubulin remained unchanged in the highly resistant P388/4.0r-M cells (Fig. 2). Nevertheless, it remains unclear whether the ratio of the polymerized tubulin/total tubulin is relevant to the drug-resistance phenotype.

There are a few reports demonstrating that microtubule dynamics are influenced by the β-tubulin isotypic composition.[11, 13, 14] We examined the specific β-tubulin isotype composition by competitive RT-PCR. This method has several well-established advantages over other methods of RNA quantification utilizing RT-PCR. Since the quantification procedure is based on the ratio between the amounts of competitor and target products, the technique is unaffected by the overall yield of either the RT or PCR steps.[15–17] To obtain isotype-specificity, we chose antisense primers from 3′-untranslated regions. The expression of β2 isotype in the resistant cells was equivalent to that in the parental cells. Isotype 4 was found at very low levels in both parental and resistant cell lines. The remaining two isotypes, β1 and β6 were not detected by this RT-PCR method in parental or resistant cell lines. Haber *et al*. also found that PCR products for β1, β4 and β6 isotypes could not be detected by RT-PCR.[5] Isotype-specific expression of β-tubulin has been suggested to occur in malignant tumor cells, and this may be related to the antitumor effect of antimitotic agents, especially β-tubulin binders. For this reason, it will be necessary to examine the patterns of β-tubulin isotype expression in tumor tissue samples. This competitive RT-PCR method is convenient for the analysis of the isotype-specific expression of tubulin, even in clinical materials. We found a 2-fold increase in the β5 isotype in P388/4.0r-M cells and a 0.5-fold decrease in β3 isotype in both E7010-resistant cell lines by using competitive RT-PCR (Fig. 3B). The increase in the β5 isotype may contribute to the increase of total tubulin content observed in P388/4.0r-M cells. However, it is impossible to explain the increased level of β-tubulin expression in P388/0.5r-D cells. In addition, it is considered that an increase of target molecules in cells should cause increased sensitivity of the cells to the drug. Therefore, it is unlikely that increased E7010 binding to tubulin in resistant cells plays a role in the mechanism of resistance. Although the functional effects of differences in tubulin isotype content have not been fully clarified, Banerjee *et al*.[7] showed that alteration of the relative amounts of the different tubulin isotypes changes the microtubule dynamics.[18, 19] Photoaffinity labeling of fusion proteins by labeled E7010 revealed that E7010 preferentially binds to the β3 isotype. The β-tubulin isotypes therefore may have different affinities to various anticancer drugs despite the very high level of homology between the isotypes. Each fusion protein contains two colchicine binding sites[20, 21] and the binding of E7010 to β3 fusion proteins was subject to competition by cold colchicine (Fig. 5C). These results suggest that E7010 binds preferentially to the colchicine binding site of β3-tubulin. Although the amino acid sequences around the colchicine binding sites of β-tubulin are very similar, it is likely that the individual conformational differences affect the binding affinity for E7010. In the previous report,[1] it was demonstrated that E7010 accumulates in P388 and its E7010-resistant cell lines. It is considered that E7010 exists intracellularly in a large excess in molar ratio to β3 tubulin, and therefore, reduction of β3 tubulin in resistant cells results in resistance to E7010. From this point of view, the decreased level of β3-tubulin expression in the resistant cell lines may play an important role in the resistance to E7010. To confirm this, experiments using a β3-tubulin cDNA transfectant are in progress.

The fusion proteins we used contain two of the three colchicine binding sites at the C-terminus (including residues Cys^{239}, Cys^{354}), but lack the colchicine binding site at the N-terminus. It will be important to elucidate the β-tubulin isotype-specific binding of various antimitotic agents, and these fusion proteins may enable the simple screening of antimitotic agents for isotype-specific binding.

Recently, an M40 isotype mutation (equivalent to mouse β5 isotype) was demonstrated in human paclitaxel-resistant cells.[22] We did not examine the binding capacity of β3 isotypic fusion proteins that originated from the E7010-resistant cells. We observed no mutations in the β3 isotype cDNA from E7010-resistant cells (data not shown), but further studies are desirable to screen these resistant clones for mutations in the β-tubulin genes.

In this experiment, the difference in the resistance of the two resistant clones could not be explained by the altered expression of the β3 isotype alone. A specific event, such as tubulin gene mutation, is a possible mechanism to explain the high resistance to E7010 in the highly resistant clone, P388/4.0r-D.

In conclusion, we have characterized P-glycoprotein-negative E7010-resistant cell lines. The tubulin β3 isotype, which preferentially binds to E7010, was decreased in the resistant cell lines. The altered expression of β-tubulin isotypes may contribute to the acquisition of E7010 resistance. Our results suggest that this analysis using β-tubulin isotype-specific proteins is a useful approach to study the specificity of the action of antimitotic drugs.

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