Resistance to Microtubule-Targeted Cytotoxins in a K562 Leukemia Cell Variant Associated with Altered Tubulin Expression and Polymerization

Charles Dumontet, Jean-Pierre Jaffrezou, Etsuko Tsuchiya, George E. Duran,

Gang Chen, W. Brent Derry, Leslie Wilson, Mary Ann Jordan, and Branimir I. Sikic

Department of Medicine, Divisions of Oncology and Clinical Pharmacology, Stanford University Medical Center, Stanford, CA 94305 (C. D., J.-P. J., G. E. D., G. C., and B. I. S.), and Department of Biological Sciences, University of California Santa Barbara, Santa Barbara, CA 93106 (E.T., W. B. D., L. W., and M. A. J.)

This work was supported by American Cancer Society Grants DHP-76 (B. I. S.) and DHP-43 (L. W.), Department of the Army Grant DAMD 17-94-J-4352, NIH Grants CA-52168 (B. I. S.) and CA-57291 (M. A. J.), l'Association pour la Recherche sur le Cancer , La Ligue Nationale Contre le Cancer and the Ligue Contre le Cancer de la Dr™me, and the Philippe Foundation, Inc. (C. D. and J. P. J.)

The abbreviations used are: IC_{50}, inhibitory concentration 50%; MDR, multidrug resistance; P-gp, P-glycoprotein; rt-PCR, reverse transcriptase-polymerase chain reaction

[ABSTRACT]

A vinblastine resistant cell line, KCVB2, was established by co-selecting the parental erythroleukemic cell line K562 with step-wise increased concentrations of vinblastine (Velban¨) in the presence of the cyclosporin D analogue PSC 833 (2 μM), a potent modulator of the multidrug resistance phenotype. KCVB2 cells are 8-fold resistant to the selecting agent, vinblastine, but also exhibit significant resistance to other vinca alkaloids, including 14-fold resistance to vinorelbine, as well as 6-fold cross-resistance to paclitaxel. Doubling time and morphology were similar to the parental K562 cells. Rt-PCR analysis revealed no alterations in the expression of the *mdr*1 and MRP genes. Intracellular vinblastine accumulation was unchanged. Disruption of the mitotic spindles and multiple mitotic asters occurred in both cell lines but required higher concentrations of vinblastine in KCVB2 cells than in K562 cells. Significant differences were observed in the tubulin content of KCVB2 cells: reduction of total tubulin content, increased polymerized fraction of total tubulin, and overexpression of class III beta-tubulin which is expressed at very low levels in the parental K562 cells. K562 cells transfected with murine class III beta-tubulin did not display the resistance pattern observed in KCVB2 cells. Revertants of KCVB2 manifested reversion to parental drug sensitivity, an increase in total tubulin level, and a decrease in polymerized tubulin. In conclusion, the KCVB2 cell line displays a novel mechanism of resistance to both depolymerizing and stabilizing microtubule-targeted cytotoxins which does not involve altered cellular drug accumulation, but corresponds to alterations in the total tubulin content and polymerization status, and may involve an effect on microtubule dynamics.

[Introduction]

Vinblastine (Velban^{R}) is a tubulin-binding cytotoxin with high levels of activity in a number of neoplasms, including breast cancer (1, 2). Vinca alkaloids also display significant activity in hematological malignancies (3-7). Vinblastine and its congeners, vincristine and vinorelbine, are drugs that, at high concentrations, inhibit microtubule polymerization and depolymerize microtubules. Their antitumor activity has been thought to result from destruction of spindle microtubules required for mitosis and subsequent cell division. In contrast to the vinca alkaloids, the taxanes paclitaxel (Taxol^{R}) and docetaxel (Taxotere^{R}) stimulate microtubule polymerization and stabilize microtubules. These effects have been considered to be responsible for the potent antitumor activity of paclitaxel (8).

However, recent evidence indicates that, at their lowest effective concentrations, the vinca alkaloids and paclitaxel share a common mechanism of disruption of microtubule behavior and function. The most sensitive action of both vinblastine and paclitaxel on microtubules is inhibition (stabilization) of the rapid excursions of growing and shortening at microtubule ends, called dynamic instability (9, 10). Microtubule dynamic instability is now recognized as one of the most important characteristics determining microtubule function in cells, particularly in mitosis (11). Stabilization of microtubule dynamics by low concentrations of vinblastine or paclitaxel leads to slowing or blockage of the cell cycle at mitosis which has been shown to result in cell death by apoptosis in HeLa cells (10, 12, 13).

Reduced sensitivity of neoplastic cells to microtubule-targeted agents may result from a number of factors, including reduced binding of the drugs to tubulin and/or microtubules, altered dynamic properties of the microtubules, and/or reduced drug accumulation due to increased drug efflux. Analysis of the factors involving tubulin and/or microtubules which may lead to reduced sensitivity to microtubule-targeted agents is complex. Indeed, alterations involving either the spontaneous dynamic behavior of microtubules or the binding affinity for tubulin-binding compounds may be involved (14). Alterations in tubulin isotype composition or polymerization status have been reported in cell line models resistant to various microtubule-targeted agents (15-18).

Vinblastine, like other vinca alkaloids, is a substrate for the P-glycoprotein (P-gp) efflux protein responsible for the classical "multidrug resistance" (MDR) phenotype. Cells which display this phenotype are resistant to a number of structurally unrelated compounds such as vinca alkaloids, taxanes, epipodophyllotoxins, and anthracyclines (8, 19-21). In order to identify mechanisms of drug resistance other than MDR, we have isolated a drug resistant cell line derived from the human leukemic line K562 by co-selection with incremental increases in vinblastine concentration in the presence of the MDR modulator PSC 833 (22, 23). The design of these selection experiments was to suppress the emergence of P-gp expressing mutants using PSC 833, in order to allow cells with other mechanisms of drug resistance to survive (24). The KCVB2 line displays a wide range of resistance to microtubule-active agents but not to other MDR-related drugs. Our results suggest that alterations in the tubulin content and polymerization status in the KCVB2 cells are involved in this resistance phenomenon.

[MATERIAL AND METHODS]

Drugs and Reagents. PSC 833 was kindly provided by Sandoz Pharmaceutical Corporation (Basel, Switzerland). Vinblastine (Velban^{R}) was obtained from Eli Lilly. Paclitaxel (Taxol^{R}) was obtained as a gift from the Bristol Myers Squibb Company. These drugs were dissolved in absolute ethanol at 1 mM and stored at -20oC. All other chemicals as well as the pan β tubulin monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Purified TUJ1 monoclonal antibody directed against class III β tubulin was a kind gift of Antony Frankfurter (University of Virginia).

Cell Culture. The human erythroleukemic cell line K562 was purchased from the American Type Culture Collection. The K562/R7 cell line, derived by selecting K562 cells for resistance to doxorubicin was used as an MDR positive control (25). The KCVB2 cell line was derived by exposure of the K562 cell line to increasing concentrations of vinblastine (0.1-4 nM) in the continuous presence of 2 μM PSC 833 for 18 months, then maintained with a periodic 72-hour challenge with 2 nM vinblastine in the presence of 2

µM PSC 833 every two months. This time interval was chosen because cells progressively reverted to sensitivity after a period of 2 to 3 months. All experiments were performed using cells which had been reselected one to six weeks earlier. The K562/PSC cells were obtained by continuous exposure to 2 µM PSC 833 alone. KCVB2 revertant cells were obtained by prolonged growth (> 3 months) in the absence of drug. All cell lines were cultured in McCoy's 5A medium supplemented with 10 % newborn calf serum, 2 mM glutamine, 200 units/ml penicillin/ml and 100 µg/ml streptomycin (all from Irvine Scientific, Santa Ana, CA). Cells were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2. Cell stocks were screened routinely for *Mycoplasma* by the DNA hybridization method (Gen-Probe, Inc., San Diego, CA) and by rt-PCR.

Growth Inhibition Assay. Approximately 15,000 cells per well were seeded in 96-well round-bottomed plates and incubated with and without drugs for 72 hrs at 37 ¡C in an atmosphere of 5% CO_2. Growth inhibition was evaluated by the MTT colorimetric method on triplicate assays as previously described (26). The absorbance was quantitated with a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). The IC_{50} (drug concentration resulting in 50% inhibition of MTT dye formation, compared to controls) was determined directly from semilogarithmic dose-response curves.

Drug Accumulation Assays. Intracellular vinblastine accumulation was quantitated using radiolabeled drug as previously described (20). Briefly, cells were incubated at 37oC in serum-free McCoy's 5A media supplemented with 40 mM Hepes buffer in the presence of 50 nM [^{3}H]-vinblastine (19 Ci/mmol, NEN-Dupont). After 1 hour, approximately 1.5 x 10^{6} cells were centrifuged through Versilube F50 silicone oil (General Electric Co. Waterford, NY) at 12,000 rpm for 1 min. The media and oil were aspirated, and the cell pellet solubilized in 4% SDS at 65 oC for 1 hr. Ecolite scintillation cocktail (ICN Biochemicals, Costa Mesa, CA) was added and radioactivity counted and normalized to protein content.

Reverse Transcriptase Polymerase Chain Reaction (rt-PCR). Total RNA extraction and rt-PCR were performed as previously described (27). PCR was performed in a Perkin-Elmer Cetus DNA 9600 Thermal Cycler (Norwalk, CT) using the following profile: 10 sec at 94oC, 30 sec at 55oC, and 30 sec at 72oC. The amplimers used in this study were synthesized by Operon Technologies (Alameda, CA). Amplimers for *mdr*1 were: 3020-3037 (forward) and 3168-3187 (reverse). MRP (Multidrug Resistance Associated Protein) amplimers were: 2954-2974 (forward) and 3069-3086 (reverse). 28S ribosomal cDNA (1501-1520 forward; 1846-1826 reverse) was used as an endogenous internal control (27).

We designed the following primers for analysis of the β-tubulin isotypes (Arabic numerals refer to the gene, Roman numerals refer to the tubulin protein isotype class):

- M40 (class I) forward primer: (-42, -23) CCA TAC ATA CCT TGA GGC GA

- M40 reverse primer: (227, 246) GCC AAA AGG ACC TGA GCG AA

- β9 (class II) forward primer: (1131, 1150) CGC ATC TCC GAG CAG TTC AC

- β9 (class II) reverse primer: (1301, 1319) TCG CCC TCC TCC TCC TCG A

- β4 (class III) forward primer: (1, 15) ATG AGG GAA ATC GTG

- β4 (class III) reverse primer: (223, 243) AAA GGC CCC TGA GCG GAC ACT

- 5β (class IVa) forward primer: (-85, -68) TCT CCG CCG CAT CTT CCA

- 5β (class IVa) reverse primer: (167, 186) TCT GGG GAC ATA ATT TCC TC

- β2 (Class IVb) forward primer: (-42, -23) GTC TAC TTC CTC CTC TTC CC

- β2 reverse primer: (281, 300) GTT GTT CCC AGC ACC ACT CT

These primers were designed using published sequence data for M40, 5β and β2 isotypes or, in the case of β4 isotype, a consensus forward primer and partial sequence information generously provided by Kevin Sullivan (Scripps Research Institute, La Jolla, CA) (28). These primers were designed to span introns. In the case of class II isotype, sequence was provided by screening expressed sequence tags (EST T03799) from the EMBL GeneBank, using the peptide sequence previously reported by Cowan et al. (29).

PCR samples were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and analyzed by densitometric reading of bands on an Alpha Innotech IS-1000 image analyzer (San Leandro, CA). To ensure that samples were in the linear range of amplification, PCR was performed using both various dilutions of cDNA and various numbers of cycles of amplification. In separate experiments, the presence of intronless pseudogenes was monitored both by performing PCR on RNA preparations without reverse transcription, and on cDNA reverse transcribed from DNAse-treated RNA (MessageClean, GenHunter, Brookline, MA).

Evaluation of total tubulin, the class III tubulin isotype, and polymerized tubulin, by immunoblotting. Cells were harvested in log phase of growth and pellets were resuspended in lysis buffer containing Tris-HCl pH 6.80, $MgCl_2$ 1mM, 2 mM EGTA, 0.2% Tween 20 and protease inhibitors (PMSF 1 mM, leupeptin 50 µg/ml, pepstatin 1 µg/ml, trypsin inhibitor 1 mg/ml and aprotinin 20 µg/ml (Sigma, St. Louis, MO)). Total protein was quantified by the Lowry assay (30) and samples containing 50 or 100 µg of protein were prepared in SDS then boiled before being applied to a 12% polyacrylamide gel, as previously described. Proteins were then blotted onto a Hybond-ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) using a Bio-Rad semi-dry blotter (Hercules, CA). The membrane was blocked with buffer containing 5% milk and 1% bovine serum albumin, then incubated 2 hours at room temperature with pan-§ anti-tubulin monoclonal antibody (1:3000 dilution) or anti-class III β-tubulin isotype monoclonal antibody TUJ1 (1:1000 dilution), washed three times, incubated with goat anti-mouse antibody, washed, incubated in streptavidin-biotin, washed and processed with ECL reagents. Phosphocellulose purified tubulin prepared from bovine brain was used as a control. Preliminary experiments were performed to determine the concentration of antibodies allowing quantification of tubulin content.

Polymerized and soluble tubulin were distinguished using a modified version of the method reported by Cabral et al. (15). Briefly, cells were harvested, washed and lysed as described above. Cell lysates were then incubated for 5 min at 37 °C in the dark, and the cellular residues mixed briefly and centrifuged at 14,000 rpm for 10 min at room temperature. The resultant supernatants were carefully

transferred to a separate centrifuge tube and kept on ice. The pellet was resuspended in Ling's Lysis buffer (10 mM Tris pH 7.5, 1.5 mM $MgCl_2$ and 10 mM KCl) in a volume equal to the supernatant. A volume equivalent to 100 µg of sample was incubated in 4 X Laemmli buffer, vortexed, boiled for 10 min prior to loading onto a 12% acrylamide-SDS gel. Gels were then processed for immunoblotting as described above. The percent of polymerized tubulin was calculated by comparing the relative ratio of polymerized tubulin (pellet fraction) to the unpolymerized (supernatant) tubulin dimers by densitometry.

Immunofluorescence microscopy. Log phase cells, grown in the presence or absence of vinblastine for 24 hours, were collected by centrifugation (1,000 rpm, 3 min.) and resuspended in 5 ml of 10% formalin, 2 mM EGTA in phosphate-buffered saline (PBS) pH 7.4. Following fixation for 10 min the cells were centrifuged and resuspended in 5 ml of 99.6% methanol, 2 mM EGTA (-20 ^{O}C) for 10 min. Following three washes with PBS containing 0.1% bovine serum albumin (PBS-BSA), nonspecific antibody binding was blocked using normal goat serum (1 part serum to 4 parts PBS-BSA, 15 min) and cells were then resuspended for 1 hour at 37 ^{O}C in a mouse monoclonal antibody specific for β-tubulin (a gift from Dr. Michael Klymkowski, University of Colorado, Boulder) (1:500 in PBS-BSA). Following three washes with PBS-BSA, cells were stained with a fluorescein isothiocyanate-conjugated-goat-anti-mouse IgG for 1 hour at 37 ^{O}C. DAPI (4,6-diamino-2-phenylindole, 1 µg/ml, Sigma) was added to the cell suspension one minute prior to centrifugation and washing (three times with PBS-BSA). Fifty µl of fixed stained cells were mounted on glass slides with approximately 30 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Photomicrographs were obtained using a Zeiss photomicroscope III equipped with epifluorescence.

Transfection of K562 cells with class III β tubulin. K562 cells were transfected with class III β tubulin. In the absence of available human cDNAs for class III β tubulin, we used murine class III (mβ6 gene) to construct a transfection vector. Murine mβ6 shares 99% homology with human class III β tubulin at the amino acid level (31). Murine class III gene was excised from a pGEM/2Z vector (generously

provided by Juan C. Zabala, Universidad de Cantabria, Santander, Spain) with *Eco*RI and transferred to a pBK/CMV vector (Stratagene, La Jolla, CA). Orientation and integrity of the vector was confirmed by sequencing (Sequenase Quick Denature Plasmid Sequencing Kit, USB, Cleveland, OH). K562 cells were transfected by lipofection (Lipofectin, Sigma) of 10 μg of *Ssp*-1 linearized plasmid into 20 x 10^6 cells, following the manufacturer's instructions. The resulting stable transfectants, designated K562/mβ6 cells, were isolated by incubation in G418 (400 μg/ml) and amplified for further characterization. As a control, K562 cells were transfected with pBK/CMV vector with no insert.

[RESULTS]

Resistance profile of vinblastine-selected KCVB2 cells. The sensitivity of K562/PSC and KCVB2 cells to several cytotoxic drugs is shown in Table 1. Compared to K562 cells grown in PSC only, KCVB2 cells presented significant resistance (8-fold) to the selecting agent vinblastine. These cells also displayed cross-resistance to a number of microtubule-active agents such as other vinca alkaloids and paclitaxel. The highest degree of resistance was observed with vinorelbine, a derivative of vinblastine. Conversely K562/m β6 cells, which expressed class III β-tubulin, did not display resistance to microtubule-targeted agents when compared to K562 cells transfected with the pBK/CMV vector only. KCVB2 revertant cells had a drug sensitivity phenotype comparable to that of the K562/PSC cells (data not shown). The doubling time of KCVB2 cells was comparable to that of K562 cells (22 hours).

Intracellular vinblastine concentration. The cellular accumulation of [^{3}H]-vinblastine was examined in K562/PSC, vinblastine-selected KCVB2, and MDR positive K562/R7 cells. Whereas, as expected, K562/R7 demonstrated significantly decreased accumulation of [^{3}H]-vinblastine, no significant differences were observed in [^{3}H]-vinblastine levels between KCVB2 and K562/PSC cells (Figure 1).

Analysis of *mdr*1, MRP and β-tubulin isotype expression by rt-PCR. Total RNA was extracted from the cell lines and was analyzed by PCR for the presence of *mdr*1 transcripts. As shown in Figure 2, K562 cells and KCVB2 cells had very low levels of *mdr*1 transcript, contrary to K562/R7, a P-gp-positive cell line which was derived from K562 and used as a control. No overexpression of the MRP gene was observed in KCVB2 compared to the parental cells.

Tubulin isotypes share a considerable degree of homology at the amino acid level, but are sufficiently divergent at the nucleotide level to allow the design of specific primers. Moreover, 3' untranslated regions differ considerably between isotypes and were used to design primers whenever these data were available. In all cases, the PCR products had the expected size and were further confirmed to be isotype-specific by sequencing (Sequenase PCR Product Sequencing Kit, Cleveland, OH). All of the PCR products produced with the primers reported in this publication have been sequenced and

unambiguous sequences obtained, which were identical to the previously reported sequences. In the case of the β4 reverse primer, the amino acid sequence was deduced and compared to other available class III sequences (chicken cβ4 (31) and murine mβ6, provided by S.A. Lewis). The human β4 PCR product was shown to contain the same isotype-specific amino acid differences as the class III described in mouse and chicken.

Analysis of untranscribed RNA and DNAse-treated transcribed RNA indicated that the only set of primers yielding PCR products corresponding to pseudogenes were the 5β primers. In this case however, the products obtained represented a small fraction (less than 5%) of the corresponding products obtained from cDNA, and pretreatment with DNAse did not significantly modify the amount of PCR product obtained (data not shown).

Analysis of β-tubulin isotype transcripts by semi-quantitative rt-PCR on four different batches of cells demonstrated comparable levels of most β-tubulin isotype transcripts (classes I, II, IVa and IVb) in K562/PSC cells and KCVB2 cells. KCVB2 cells displayed 3-fold higher expression of transcripts for the class III isotype (β4) than the K562/PSC cells (Table 2 and Figure 3). The levels of β-tubulin transcripts in the revertant KCVB2 cells were comparable to those of the resistant KCVB2 cells. In particular the levels of class III transcripts in revertant KCVB2 cells remained higher than those observed in K562/PSC cells.

Total tubulin and class III tubulin content. Analysis of total β tubulin content by immunoblotting of total cell lysates with pan-β tubulin monoclonal antibody revealed significantly reduced amounts of total tubulin in KCVB2 cells in comparison to K562/PSC cells (Figure 4). Quantification of immunoblots by densitometry showed that the total β-tubulin content of KCVB2 cells was approximately 30% that of the K562/PSC cells. Identical results were obtained by fluorescence activated cell sorting of permeabilized cells with a pan-β monoclonal antibody (data not shown). Revertant cells, which were derived from KCVB2 cells by prolonged culture in the absence of drug, and which had the same drug sensitivity phenotype as the K562/PSC cells, had higher tubulin levels than resistant KCVB2 cells.

In keeping with the PCR results described above, KCVB2 cells were found by immunoblotting to express class III tubulin protein more strongly than K562/PSC cells (Figure 4). K562/mβ6 cells

overexpressed class III β tubulin protein but had a total tubulin content comparable to that of K562 cells transfected with pBK/CMV vector only.

Analysis of the polymerized status of tubulin showed that a significantly larger fraction of total tubulin was polymerized (74%) in KCVB2 cells than in K562/PSC cells (37%) (Figure 5). In the revertant KCVB2 cells however, this fraction was found to be similar to that of the K562/PSC cells.

Effects of vinblastine on mitosis, spindle morphology and nuclear morphology. Vinblastine has been found to arrest mitosis specifically at the transition from metaphase to anaphase in HeLa and BSC cells. Mitotic block by vinblastine in these cells is associated with abnormal spindle organization and chromosomes that do not congress properly to form the metaphase plate (12, 32-34). Strong evidence suggests that the mitotic block and the accompanying spindle abnormalities result from the suppression of microtubule dynamics by low concentrations of vinblastine. Thus we examined the effects of a range of vinblastine concentrations on mitotic arrest and microtubule, chromosome, and nuclear organization in K562 and KCVB2 cells by immunofluorescence microscopy.

Both K562 cells and KCVB2 cells were arrested in mitotic metaphase by vinblastine, although K562 cells were arrested at lower vinblastine concentrations than KCVB2 cells. For example, after incubation of cells with 10 nM vinblastine for one cell cycle (24 h), 40% of K562 cells were in metaphase whereas only 20% of KCVB2 cells were in metaphase. The block occurred specifically at the transition from metaphase to anaphase. In K562 cells, no cells were in anaphase at vinblastine concentrations >10 nM, whereas with KCVB2 cells, anaphase did not occur at vinblastine concentrations >30 nM (Table 3).

The morphology of K562 cells was more severely altered by vinblastine than the morphology of the vinblastine-resistant KCVB2 cells. For example, after incubation with 10 nM vinblastine, mitotic spindle microtubules of K562 cells were largely depolymerized; only small remnants of microtubules remained. All spindles (100%) were abnormal and were generally small monopolar aggregates of microtubules enclosed in disorganized masses of condensed chromosomes (data not shown). In contrast, the morphology of KCVB2 cells was less affected by vinblastine. Microtubules were longer and their total mass appeared greater, and some spindles (20%) were normal bipolar spindles with a compact

metaphase plate of chromosomes (data not shown). Of the KCVB2 cells remaining in interphase, 30% were multinucleate. Thus although the morphology of both cell lines was the same in the absence of vinblastine, low concentrations of vinblastine altered microtubule morphology to a greater degree in K562 cells than in vinblastine-resistant KCVB2 cells.

[DISCUSSION]

Resistance to vinblastine and other vinca alkaloids has been associated with the multidrug resistance (MDR) phenotype mediated by the multidrug transporter, P-gp (35-37). There is increasing evidence that expression of the *mdr*1 gene in neoplastic cells confers an adverse prognosis in leukemias, lymphomas, and some childhood malignancies (31, 32, 34-39). These data have spurred clinical trials attempting to reverse MDR by combined therapy using modulators (21, 39-44).

Drug resistance in cancer cells is often multifactorial, and any attempt to negate one mechanism of resistance will probably lead to the emergence of alternate mechanisms, albeit at a lower frequency. We have thus shown by fluctuation analysis that after stringent exposure to doxorubicin, all of the surviving human sarcoma cell variants displayed the MDR phenotype (27). However, when cells were exposed to doxorubicin in the presence of the MDR modulator PSC 833, the occurrence of resistance was 10-fold less frequent (24). All of these variants were MDR-negative but displayed topoisomerase II alterations. In a similar approach, we demonstrated that MES-SA cells surviving stringent exposure to paclitaxel displayed the MDR phenotype in 45% of cases, and contained reduced expression of 5§ and §4 tubulin isotype transcript (7).

In the case of microtubule-active agents, such as vinca alkaloids, it has been suggested that alterations in tubulin and/or in microtubule dynamics are involved in resistance to these drugs (14, 15). In order to investigate non-MDR mechanisms of resistance to vinca alkaloids, we chose to select vinblastine-resistant cells in the presence of the potent MDR modulator, PSC 833. We thus obtained the KCVB2 cell line, an MDR-negative variant of K562 which displays broad cross-resistance to tubulin active agents, including both depolymerizing and stabilizing drugs. The KCVB2 cells do not have increased

levels of MRP nor do they display an accumulation defect for [^{3}H]-vinblastine, which suggests that they do not possess a drug transporter different from P-gp. However KCVB2 cells possess striking alterations of their tubulin content. In comparison to control cells grown in PSC 833 alone, these cells have a reduced total tubulin content, contain a significantly higher fraction of polymerized tubulin, and overexpress class III §-tubulin.

Human genes coding for α- and β-tubulin constitute a multigene family of approximately 15 to 20 members, including several intronless pseudogenes (45, 46). This is an important factor to consider when analyzing tubulin gene expression by rt-PCR, and alterations displayed at the transcript level should be confirmed when possible at the protein level. The functional significance of tubulin isotypes remains a subject of controversy (47, 48). Little is known regarding the interactions between specific tubulin isotypes and microtubule-active agents (49-51). We have shown that a paclitaxel-resistant variant of K562 specifically overexpresses class IVa β-tubulin (52). Others have shown alterations in tubulin isotype expression in cell lines selected with various microtubule specific cytotoxins (16, 53). Kavallaris et al. have shown overexpression of tubulin isotypes in ovarian tumors resistant to taxanes (54).

Human class III β-tubulin cDNA was isolated by Sullivan and Cleveland from a human placental library (55). Analyses of expression of class III in vertebrates show it to be a minor neuronal isotype. Class III isotype expression and phosphorylation has been shown to be associated with differentiation in a mouse neuroblastoma cell line, and accumulation of class III β-tubulin was observed during neurite outgrowth in a rat model (56, 57). Analysis of class III β-tubulin expression during chick embryogenesis has shown that the content of this isotype increases in conjunction with the rate of neuronal differentiation and that it exists in a number of isoforms, suggesting posttranslational modification (50, 58). More recent data has shown that the class III β-tubulin isotype has unique dynamic features. Falconer et al. have reported that class III is preferentially found in colchicine-labile microtubules (59). Panda et al. have demonstrated that microtubules assembled from purified $\alpha\beta_{III}$ dimers were more dynamic than those assembled either with $\alpha\beta_{II}$ or $\alpha\beta_{IV}$ (60). Colarusso et al. have recently reported a paclitaxel-resistant prostate line which ov erexpresses class III β tubulin (61). Laferriere et al. have shown that paclitaxel promotes post translational modification of class III tubulin (62). These data suggest that the

class III β tubulin isotype may be involved in drug resistance mechanisms because of its unique dynamic features.

KCVB2 cells have a reduced total tubulin content and a higher polymerized tubulin fraction. However our rt-PCR results indicate that the transcript levels of tubulin isotypes are comparable to those observed in the sensitive K562/PSC cells. This suggests that there is a post-transcriptional regulatory mechanism in KCVB2 cells which affects the protein levels of β-tubulin isotypes. It has previously been reported that the synthesis of tubulin subunits is strictly regulated, possibly by translational or post-translational mechanisms. Cleveland et al. have described an autoregulatory mechanism at the translational level, involving the first four amino acids of tubulin, which are common to all known isotype (55, 63). More recent data supports the notion that adequate folding of tubulin subunits requires appropriate chaperone molecules, which act as regulators of tubulin function *in vitro*. (64, 65).

In an attempt to determine the respective roles of the various tubulin alterations in the occurrence of resistance in KCVB2 cells, we transfected parental K562 cells with the murine class III β-tubulin gene. These cells were found to express class III tubulin isotype protein at levels comparable to those found in KCVB2 cells, with total tubulin levels comparable to K562 cells. These cells did not display resistance to tubulin active agents. Although the gene used for these transfection experiments was murine, one possible interpretation is that the expression of class III β-tubulin may not be sufficient by itself to induce resistance to microtubule-active cytotoxins. Thus the mechanism of resistance in KCVB2 cells may involve the reduction in total tubulin or the stoichiometry of class III β-tubulin. This is supported by the fact that revertant cells, which display the same sensitivity phenotype as parental cells, have higher total tubulin content and a smaller polymerized fraction than resistant KCVB2 cells. Similar to our results with KCVB2 cells, reduction in total tubulin levels in drug-resistant mutants has been associated with increased taxol resistance (14, 66). However, in contrast to our results with KCVB2 cells, these authors found that in most cases mutant cells that are resistant to paclitaxel are hypersensitive to microtubule-destabilizing drugs, and cells that are resistant to microtubule depolymerizing agents such as vinblastine and colchicine are hypersensitive to paclitaxel.

Thus, it is interesting that KCVB2 cells were approximately equally resistant to both paclitaxel and to the vinca alkaloids (vinblastine, vincristine, and vinorelbine), and that they were also resistant to colchicine (Table 1). These three classes of drugs each bind to a different site on tubulin. Thus the mechanism of resistance is not likely to result primarily from an alteration in their binding sites. The simultaneous resistance to three classes of microtubule-active drugs that act most potently by stabilizing microtubule dynamic instability (9-12) strongly suggests that the basis of resistance to all three classes of microtubule-binding drugs may be a compensatory increase in the dynamics of the microtubules of the KCVB2 cells. The presence of class III isotype of tubulin ($\alpha\beta_{III}$) in the microtubules of the resistant cells may induce such an increase in their dynamic instability. Microtubules composed of purified bovine brain class III tubulin isotype ($\alpha\beta_{III}$) are considerably more dynamic than microtubules made from purified $\alpha\beta_{II}$ or $\alpha\beta_{IV}$-tubulin isotypes or from unfractionated tubulin (60). In addition, the dynamics of isotype mixtures are not strictly predictable from knowledge of the dynamics of the purified isotypes. Thus the presence of $\alpha\beta_{III}$ in a background of a large (70%) reduction in total tubulin in KCVB2 cells might lead to a significant increase in microtubule dynamics and resistance to microtubule stabilizing drugs such as vinblastine and paclitaxel whereas the presence of $\alpha\beta_{III}$ in the background of normal levels of total tubulin in the transfected K562 cells might prove insignificant. However the data concerning dynamic properties of individual istoypes obtained using purified tubulin must be interpreted with caution and may not be relevant to the situation existing within intact cells.

Circumvention of the MDR phenotype by potent modulators is presently an achievable goal. However drug resistance is usually multifactorial and in the case of microtubule-active agents, alterations in microtubule components and dynamics are prime candidates for second-line resistance mechanisms. Our data suggest that broad cross resistance to microtubule-targeted agents may result from altered tubulin content. The occurrence of this phenomenon in the clinic as well as the precise role of tubulin isotypes in resistance to these agents remain to be determined.

[Acknowledgments]

The authors thank Katherine A. Steger for help in performing the rt-PCR experiments and S. A. Lewis and N. Cowan for information concerning and subclones of human β-tubulins.

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[Tables]

Table 1. Drug-Resistance Phenotype in K562, KCVB2 and K562/mβ6 cell lines

Drug	K562/PSC IC50 nM	KCVB2#	K562/mβ6#*
Vinblastine	5.3 + 3.6	8	1
Vincristine	3.0 + 1.9	5	1
Vinorelbine	1.0 + 0.8	14	1
Paclitaxel	5.5 + 0.5	6	1
Colchicine	250 + 110	2	1
Podophyllotoxin	6.3 + 1.1	1	1
Etoposide	4000 + 2300	1	1
Doxorubicin	290 + 140	1	1

The IC_{50} is the concentration of anticancer drug that inhibited cell growth by 50% in a 72hr MTT assay. # Values for the KCVB2 and K562/mβ6 lines are expressed as the ratios of the IC_{50}'s compared to the K562/PSC cells. * K562/mβ6 cells were compared *in vitro* to K562 cells transfected with pBK/CMV. These latter cells have IC_{50} values comparable to those of K562/PSC cells. Values are means of at least three independent experiments ± SE.

Table 2 - Expression of β-tubulin isotypes in KCVB2 and KCVB2 revertant cells.

β-tubulin isotype	Number of cycles	KCVB2	KCVB2 rev
M40	22	1.0 ± 0.3	0.9 ± 0.2
β9	25	0.9 ± 0.2	1.2 ± 0.4
β4	30	3.1 ± 0.3	2.2 ± 0.7
5β	42	1.1 ± 0.2	1.0 ± 0.3
β2	34	1.2 ± 0.3	1.4 ± 0.2

β-tubulin isotype expression was analyzed by semi-quantitative rt-PCR, using ribosomal RNA as an internal control; expression in KCVB2 and KCVB2 revertant cells is presented relative to expression in K562/PSC cells. PCR reactions were carried out at various cycles and at various cDNA concentrations to ensure that the reactions were not at saturation. Results shown indicate mean ± SE of at least 4 experiments with different batches of cells.

Table 3. Effects of vinblastine on metaphase/anaphase ratios after 24 h incubation.

Vinblastine (nM)	Cell in anaphase	Cells in metaphase
	K562	KCVB2
0	0.17 ± 0.02	0.23 ± 0.03
10	0	0.02 ± 0.01
30	0	0
100	0	0
300	0	0

Data are the mean ± SE of two experiments.

[Legends to figures]

Figure 1. Steady state accumulation of [^{3}H]-vinblastine.

Drug sensitive K562/PSC cells, vinblastine-selected KCVB2 cells, and the MDR cell line K562/R7 were incubated with 50nM [^{3}H]-vinblastine for 2 hrs. Values are mean of three independent experiments.

Figure 2. Analysis of *mdr*1 and MRP in K562 cells and variants.

K562/PSC cells, vinblastine-selected KCVB2 cells, and the MDR cell line K562/R7 were analyzed for *mdr*1 and MRP using reverse transcription PCR. Ribosomal RNA was used as control.

Figure 3. Analysis of expression of β-tubulin isotypes in K562 and KCVB2 cells by rt-PCR. cDNAs from K562/PSC, KCVB2 and KCVB2 revertant (KCVB2 rev) cells was analyzed by rt-PCR using primers for each β-tubulin isotypes. PCR products were run on a polyacrylamide gel, stained with ethidium bromide and analyzed by densitometry.

Figure 4. Total beta-tubulin and class III β-tubulin content in K562/PSC and KCVB2 cells. Cell lysates from log phase cells were applied to a 12% polyacrylamide gel and immunostaining was performed with pan-β tubulin monoclonal antibody or class III monoclonal antibody. KCVB2 rev: KCVB2 revertant cells.

Figure 5. Polymerized and soluble tubulin in K562/PSC and KCVB2 cells. Cell lysates from log phase cells were processed in order to separate polymerized and soluble tubulin. Fractions were analyzed by immunobloting 100 µg of sample with pan-β monoclonal antibody. Amounts of polymerized and soluble tubulin were determined by densitometric analysis of blots. The values shown are the mean $\pm$ SE of results obtained with three separate batches of cells.