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Species-Specific Differences in Taxol Transport and Cytotoxicity Against Human and Rodent Tumor Cells

EVIDENCE FOR AN ALTERNATE TRANSPORT SYSTEM?

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ABSTRACT. The efficacy of taxol against a wide range of sensitive and refractory solid tumors has prompted extensive investigation into the factors that influence its cytotoxicity. Our preliminary observations indicated that taxol had a superior antitumor effect against human cells (Daudi, K562, 2008, 2008/C13*, 2780 and C70) compared with its effect against rodent cells (WS, WR, NIH3T3, and CHO). Although verapamil, an inhibitor of P-glycoprotein function, markedly increased the efficacy of taxol against the rodent cells (WS, WR, and CHO), the expression of P-glycoprotein was found only at low levels in the WR cells. In addition, levels of the multidrug resistance-associated protein (MRP), as assessed by reverse transcriptase-polymerase chain reaction analysis, were found to be higher in the human than in the rodent cells, although MRP mRNA was not detected by northern blotting. Transport studies indicated that the reduced sensitivity of the rodent cells to taxol was due to decreased intracellular taxol levels and reduced intracellular binding. However, no correlation was found between the intracellular binding of taxol and the intracellular levels of α- and β-tubulin, or the intracellular concentration of polymerized tubulin. These studies were extended further by assessing the binding of taxol to semi-purified microtubule proteins from WS, CHO and 2008/C13* cells *in vitro*. The microtubule protein preparations from WS, CHO and 2008/C13* cells, which have a 50-fold difference in their sensitivity to taxol, were found to bind equal amounts of radiolabeled taxol, and this binding was inhibited (80%) in the presence of unlabeled taxol. These results lead us to propose the presence in the rodent cells of an alternative taxol transport system that is distinct from the P-glycoprotein and MRP systems. BIOCHEM PHARMACOL 51;3:301–311 1996.

KEY WORDS. taxol cytotoxicity; transport; differences; rodent cells; human cells

Taxol, a diterpenoid plant product [1], has demonstrated superior antitumor activity *in vitro*, and *in vivo*, against a variety of human tumors [2–4]. This has prompted extensive study of the biochemical factors affecting its cytotoxicity. Taxol acts in a unique manner; it binds preferentially to the N-terminal region of the β-tubulin subunit and shifts the dynamic, intracellular equilibrium away from tubulin dimers towards the formation of microtubules [5–13]. These highly stable microtubule polymers resist depolymerization by calcium or cold temperatures, conditions that usually promote disassembly [7, 10, 14]. The unusual stability of the microtubules blocks the cell in mitosis, which results in inhibition of cell division [2, 10, 15, 16]. The observation that cells in the mitotic phase of the cell cycle are more sensitive to taxol when compared with cells in interphase supports this model of taxol action [17, 18]. Several elegant studies have also demonstrated that taxol treatment of human leukemic cells induces the formation of microtubule bundles and asters in the inter- and mitotic-phases of the cell cycle and that these phenomena appear to correlate with the cytotoxic action of taxol [17, 19]. Furthermore, in taxol-resistant leukemic cells, microtubule bundle formation appears to be reversible and the cells become polyploid [19].

For taxol to be employed rationally in the clinic, it is important to understand the mechanisms underlying the development of the "taxol-resistance phenotype." Cross-resistance to taxol has been observed in tumors cells that exhibit the MDR† phenotype [20]. Overexpression of a 170-kDa transmembrane protein termed the P-glycoprotein, which functions as an energy-dependent drug-efflux pump, has been one of the most consistent changes observed in MDR [20]. Selection with taxol of a drug-resistant human leukemic cell population also correlated with overexpression of the P-glycoprotein, and a decreased intracellular accumulation of taxol [21]. Altered functional and molecular properties of the microtubule pro-

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† *Abbreviations*: MDR, multidrug resistance; CHO, Chinese hamster ovary; MRP, multidrug resistance-associated protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; AMV, avian myeloblastosis virus; RT-PCR, reverse-transcriptase polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PIPES, 1,4-piperazinediethanesulfonic acid; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

teins have also been implicated in the development of resistance to taxol and other antimitotic drugs [22–27]. These studies showed that the intracellular level of these drugs was similar in drug-sensitive and -resistant cells but that alterations in either the α- or the β-subunit of tubulin resulted in an altered binding affinity of the antimitotic drug for its intracellular target. Thus, the major factors that affect the efficacy of taxol appear to be the intracellular concentration of taxol and the intracellular content and nature of the polymerized tubulin.

Recent studies reported that rodent cell lines (mouse and hamster) display a markedly lower sensitivity to antimicrotubule and other natural product drugs when compared with that observed with a human cervical carcinoma (HeLa) cell line [28, 29]. Uptake of daunomycin, colchicine, and vinblastine in the rodent cells is decreased markedly as compared with that in the human cell line; uptake of taxol was not studied [28]. Verapamil was also shown to sensitize the CHO but not the human cervical carcinoma (HeLa) cells to the cytotoxic effects of different natural product drugs [29]. The response of CHO cells was comparable with that observed with a single-step selected puromycin-resistant HeLa cell clone (which displayed the MDR phenotype). These observations led the authors to suggest that the CHO cells displayed MDR, although no expression of P-glycoprotein was detected in the CHO cells and in the puromycin-resistant HeLa cell clone [29]. Lopes *et al.* [18] also showed that human ovarian carcinoma (2780) cells were more sensitive to taxol when compared with CHO cells, an observation that correlated with increased intracellular concentrations of [^{3}H]taxol in the 2780 cells [18]. Thus, the relative resistance of CHO cells to taxol appeared to be due to a defect in drug transport, but no detailed mechanism was elucidated. Roy and Horwitz [30] also observed increased expression of a phosphoglycoprotein (distinct from the P-glycoprotein), which correlated with decreased intracellular drug accumulation in a taxol-resistant murine macrophage-like cell line. The expression of this protein was not detected in the sensitive parental cells.

Thus, it is apparent that resistance to taxol can develop by several different mechanisms; the relative importance of each is difficult to assess. Furthermore, if the rodent cells have an intrinsic resistance to taxol, the biochemical cause of this effect is unknown. The present study was designed to correlate taxol sensitivity to its transport, intracellular levels of polymerized tubulin, and the presence/absence of MDR-mediating proteins, viz. P-glycoprotein and MRP [31], in a series of four rodent and six human cell lines.

MATERIALS AND METHODS

Materials

Taxol was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD. [^{3}H]Taxol (19.3 Ci/mmol) was obtained from the Research Triangle Institute, NC, through the National Cancer Institute, Bethesda, MD. Purity was checked by previously published procedures [18]. MTT, formaldehyde, and verapamil were purchased from the Sigma Chemical Co. (St. Louis, MO). Denhardt's reagent (50×) and formamide were purchased from the USB Corp. (Cleveland, OH). Sonicated salmon sperm DNA (10 mg/mL) was purchased from Stratagene (La Jolla, CA). [α-^{32}P]dCTP (3000 Ci/mol) was purchased from NEN Research Products (Boston, MA). Nytran Plus membranes were purchased from Schleicher & Schuell (Keene, NH). Immobilon-P membranes (PVDF membranes) were purchased from Millipore (Bedford, MA). GF/C glass fiber filters were purchased from Whatman LabSales (Hillsboro, OR). The Multiprime DNA labeling system was purchased from the Amersham Corp. (Arlington Heights, IL). Monoclonal antibody to α-tubulin was obtained from Oncogene Sci. (Cambridge, MA). Monoclonal antibody to β-tubulin was purchased from Boehringer Mannheim (Indianapolis, IN). Monoclonal antibody to P-glycoprotein (JSB-1) was obtained from Signet Laboratories, Inc. (Dedham, MA). The alkaline phosphatase conjugated anti-mouse rabbit monoclonal antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Human MDR1 (No. 39839), α-tubulin (No. 37795) and β-tubulin (No. 37855) plasmids were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All other chemicals, reagents, and supplies were purchased from commercial sources.

Cell and Culture Conditions

Walker rat lymphoma cells sensitive (WS) and resistant (WR) to cisplatin were obtained from Dr. J. J. Roberts (Institute of Cancer Research, Sutton, England) and were grown as described earlier. CHO, NIH3T3, Daudi, and K562 cell lines were obtained from the ATCC. The human ovarian carcinoma cells, sensitive (2008) and resistant (2008/C13*) to cisplatin, were obtained from Dr. P. Andrews (Georgetown University, Washington, DC). Other cisplatin-sensitive (2780) and -resistant (C70) human ovarian carcinoma cells were obtained from Dr. T. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The P-glycoprotein positive CHO subline (CR1R12) was obtained from Dr. Alan Senior (University of Rochester, Rochester, NY). The culture conditions for these cell lines have been described previously [17, 18, 32–36].

Cytotoxicity Assays

The *in vitro* cytotoxicity of taxol alone or in the presence of verapamil was evaluated by the tetrazolium dye assay as described previously [37]. Briefly, tumor cells were seeded in 96-well tissue culture plates and exposed to different taxol concentrations for a period of 72 hr at 37° in a 5% CO_2 atmosphere. In experiments where pretreatment with verapamil was being investigated, tumor cells were seeded into 96-well tissue culture plates and exposed to the modulator for 24 hr. Thereafter, appropriate concentrations of taxol were added, and the cells were incubated as before in the presence of verapamil for 72 hr. A solution of MTT (5 mg/mL) was prepared 6 hr before the end of the exposure period, 20 μL was added to each well, and the plates were further incubated at

37° in a 5% CO_2 atmosphere. At the end of the exposure period, the formazon crystals were dissolved in a solution containing 0.1 N HCl-isopropanol, and the absorbance of the colored product was measured at 570 nm in an automated microplate reader (model EL311s, BIO-TEK Instruments, Inc., Winooski, VT). Wells containing no cells were used as blanks, and wells containing cells but no drugs were used to determine the cell survival of the untreated cells.

P-glycoprotein Immunoblots

Semi-enriched plasma membrane fractions from all the cell lines were prepared by suspending cells (5×10^7) in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$ and a protease inhibitor mixture consisting of 2 mM PMSF, 2 μg/mL aprotinin, 200 μg/mL EDTA, and 1 μg/mL pepstatin. After 10 min at 4°, the cells were homogenized in a Dounce homogenizer and adjusted to 250 mM sucrose before centrifugation at 800 *g* and 4° for 20 min. The supernatant was further centrifuged at 100,000 *g* and 4° for 20 min. The pellet (membrane fraction) was resuspended in 10 mM Tris-HCl (pH 7.4), 125 mM sucrose, and the above protease inhibitor mixture. The membrane proteins (100 μg) were mixed with the solubilization buffer (4 M urea, 0.5% SDS, 50 mM DTT) and subjected to electrophoresis in a 6% polyacrylamide separating gel without heating. After electrophoresis, the separated proteins were transferred electrophoretically to PVDF membranes using a semidry blotter (Bio-Rad Laboratories, Melville, NY) and immunostained using a 1:100 dilution of the primary antibody (JSB-1). Briefly, the membranes were preincubated with a blocking solution containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS), 5% (w/v) nonfat dried milk for 1 hr at room temperature with agitation. The membranes were then incubated with the primary antibody in TBS for 2 hr. The membranes were washed with TBS containing 0.05% (v/v) Tween-20 (TBS-T) and then treated with an alkaline phosphatase conjugated secondary antibody for 1 hr. After thorough washing with TBS-T, the membranes were stained with an alkaline phosphatase detection kit (Bio-Rad).

RNA Isolation and Northern Blots

Total RNA from the rodent and human cell lines was extracted by the RNAzol method as directed by the supplier (Tel-Test, Inc., Friendswood, TX). Electrophoresis was performed under denaturing conditions using 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to a Nytran plus membrane using standard blotting techniques [38]. The membrane was baked at 80° for 3 hr and incubated with a prehybridization buffer solution [50% formamide, 10 mM sodium phosphate (pH 7.4), 5× SSC, 0.1% SDS, 5× Denhardt's reagent and 100 μg/mL sonicated salmon sperm DNA] at 42° for 4 hr. Hybridization was performed overnight at 42° in the presence of a randomly primed ^{32}P-labeled probe. The blots were washed under high stringency conditions and exposed to autoradiography films for 1–3 days at −70°.

RT-PCR Assays

Basal levels of MDR1 and MRP mRNA in the rodent and human cell lines were analyzed by the RT-PCR technique. Total cellular RNA was isolated as described previously. The synthesis of cDNA by reverse transcription was performed utilizing 1 μg of total cellular RNA, 1 mM each dNTP, 100 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 250 ng of random primers, 10 U RNAsin ribonuclease inhibitor, and 5 U of AMV reverse transcriptase, in a total volume of 25 μL. The tubes were incubated at 37° for 60 min, then at 94° for 10 min followed by quick chilling on ice. The PCR using specific primers for MDR1 [39] and MRP [40] was performed in a model 480 thermal cycler, Perkin Elmer, Cetus Corp. (Norwalk, CT), using 5 μL cDNA in a 25-μL reaction volume containing 1× PCR buffer, 250 ng of sense and antisense primers, and 1 U of Amplitaq Polymerase (Perkin Elmer).

The PCR conditions for the amplification of MDR1 were: an initial denaturation at 94° for 3 min, then 35 cycles of 94° for 30 sec, 55° for 1 min, 72° for 2 min, and a final extension at 72° for 5 min. The PCR conditions for the amplification of MRP were: an initial denaturation at 94° for 5 min, then 30 cycles of 94° for 1 min, 58° for 15 sec, 72° for 15 sec, and a final extension at 72° for 5 min. PCR products were fractionated on a 1× Tris-borate-EDTA (TBE)-3% agarose gel at 60 V for 3 hr. The gel was stained with ethidium bromide (5 μg/mL) and photographed.

[^{3}H]*Taxol Influx, Efflux and Specific Binding*

Cells (10^6 cells/mL) growing as a monolayer were allowed to adhere overnight in 24-well plates, and cells (10^6 cells/mL) growing as suspension cultures were suspended in fresh medium in a tissue culture flask and incubated at 37° in an atmosphere of 5% CO_2. In the influx studies, radiolabeled taxol (5.16 nM) was added, and the plates/flasks were incubated at 37° for the indicated time periods. The cells were then washed five times with Dulbecco's PBS (without calcium and magnesium) at 4°. Washed cells were lysed with 2 mL of 0.1 N NaOH overnight. Radioactivity was determined in a Beckman model LS 2800 liquid scintillation counter. A [^{3}H]taxol standard was counted simultaneously to determine dpm/nM taxol. The efflux studies were performed by incubating cells for 4 hr with radiolabeled taxol (5.16 nM). The cells were then washed as above and incubated in fresh medium for the indicated time at 37°. Radioactivity determinations were made as described above. The specific binding of taxol was determined by incubating cells in the presence of either radiolabeled taxol (5.16 nM) alone or with a 100-fold excess of unlabeled taxol for 2 hr. The cells were then washed, and their radioactivity was determined as described above. The specific binding was calculated by subtracting the levels of intracellular [^{3}H]taxol in the presence of unlabeled taxol, from the binding observed in the presence of radioactive taxol.

Tubulin Immunoblots

Total α- and β-tubulin levels were determined with the cell homogenate. Cells were grown in the exponential growth

phase and solubilized in the SDS-PAGE loading buffer. Extracts from 2×10^5 cells were subjected to electrophoresis using a 10% polyacrylamide gel. The separated proteins were transferred electrophoretically as described above to PVDF membranes and immunostained using α-tubulin and β-tubulin specific antibodies at a 1:100 dilution.

The concentrations of polymerized tubulin were determined in exponentially growing cells (1×10^6) that had been washed twice with phosphate-buffered saline. The resultant cell pellet was lysed in microtubule stabilization buffer [20 mM Tris-HCl (pH 6.8), 0.14 M NaCl, 0.5% (v/v) Nonidet P-40, 1 mM $MgCl_2$, 2mM EGTA and 5 μg/mL taxol] [25]. The lysate was incubated at 37° for 30 min, and then centrifuged at 39,000 *g* and 37° for 20 min. The supernatant was transferred to a fresh tube and the pellet was resuspended in the SDS-PAGE loading buffer. Extracts equal to 2×10^5 cells from the supernatant and pellet fractions obtained above were subjected to electrophoresis. The separated proteins were transferred to PVDF membranes as before and immunostained using α-tubulin and β-tubulin specific antibodies at 1:100 dilution. The intensity of individual bands from the whole cell homogenate and the supernatant and pellet fractions were determined with a Laser scanning densitometer (LKB).

Purification of Microtubule Proteins

Microtubule proteins were purified as described by Tiwari and Suprenant [41]. Briefly, tumor cells were homogenized in a buffer containing 100 mM PIPES (pH 7.4), 4 mM EGTA, 1 mM $MgSO_4$, 0.5 mM DTT and 0.1 mM PMSF at 4° for 1 hr. The supernatant was transferred to a fresh tube, and DMSO and GTP were added to final concentrations of 8% (v/v) and 1 mM, respectively. The tube was then incubated at 37° for 30 min, and the microtubules were pelleted by centrifugation at 39,000 *g* and 37° for 30 min. The microtubule pellet was incubated on ice for 30 min and resuspended by gentle homogenization in a chilled glass homogenizer in an ice-cold buffer containing 80 mM PIPES (pH 6.9), 4 mM EGTA, 1mM $MgSO_4$, 0.5 mM DTT and 0.1 mM PMSF. The assembly-disassembly procedure was repeated, and the final preparation was resuspended in 80 mM PIPES (pH 6.9), 4 mM EGTA, 1 mM $MgSO_4$, 0.5 mM DTT and 0.1 mM PMSF.

Binding of [^{3}H]Taxol to Purified Microtubule Proteins

The *in vitro* binding of [^{3}H]taxol was performed using 0.5 mg/mL of purified microtubule protein from WS, CHO and 2008/C13* cells. The reaction mixture (50 μg) contained 80 mM PIPES (pH 6.9), 4 mM EGTA, 1 mM $MgSO_4$, 0.5 mM DTT, 8% (v/v) DMSO, 1 mM GTP and increasing concentrations of radiolabeled taxol (0.1 to 10 μM). The binding reaction was carried out at 37° for 60 min. One-half of the reaction mixture was layered onto GF/C glass fiber filter papers and washed three times with cold phosphate-buffered saline. After rinsing the filters with methanol, the filters were transferred to a scintillation vial and stored overnight at room temperature. Radioactivity was measured as described in the taxol transport section.

Statistical Analysis

The regression analysis and paired *t*-test were performed using the SigmaStat Statistical Analysis System, Version 1.01.

RESULTS

Cell survival following treatment with taxol for 72 hr was determined using the MTT assay, and the IC_{50} values are presented in Table 1. The IC_{50} values were >100 nM for all the rodent cells, whereas they were <10 nM for all the human tumor cells studied (except for the 2008 cells that had an intermediate IC_{50} value of 65 nM). CHO cells with an IC_{50} value of 470 nM were most refractory to the effects of taxol.

The effect of pretreatment with verapamil on the cytotoxicity of taxol against the rodent and human cell lines was investigated. Tumor cells pretreated with verapamil for 24 hr were then exposed to taxol in the presence of verapamil for a further 72 hr. The apparent intrinsic resistance of the rodent cells to the cytotoxic effect of taxol was abrogated in three out of the four rodent cell lines by pretreatment with 10 μM verapamil (Table 1). WS, WR and CHO cells were markedly sensitized (more than 100-fold) to the cytotoxic effects of taxol when these cells were pretreated with verapamil (Table 1). In contrast, only a minimal effect was observed when the human tumor cells were pretreated with verapamil (Table 1).

Membrane proteins and total RNA from all the cell lines were analyzed for the presence/absence of P-glycoprotein by

TABLE 1. Taxol cytotoxicity and the effect of pretreatment with verapamil

Cell lines	IC_{50} (nM) Taxol	IC_{50} (nM) Verapamil + taxol	Fold-sensitization
Rodent cells			
WS	190 ± 40	1.4 ± 0.3*	136
WR	410 ± 110	0.1 ± 0.04*	4100
CHO	470 ± 47	2.8 ± 1*	168
NIH3T3	119 ± 40	67 ± 5†	1.8
Human cells			
Daudi	4.5 ± 1.5‡	7 ± 1	0
K562	8 ± 4‡	2.3 ± 1	3.5
2008	65 ± 8‡	4 ± 1†	16.3
2008/C13*	4 ± 2‡	1 ± 0.3	4
2780	5 ± 1‡	3 ± 1	1.7
C70	6 ± 2‡	4 ± 1.5	1.5

Cell survival was assessed by the MTT assay as described in Materials and Methods. The pretreatment by verapamil (10 μM) was for 24 hr, conditions that are not cytotoxic to these cells. After pretreatment, the cells were exposed to various concentrations of taxol for a further 72 hr, in the presence of verapamil. The IC_{50} value is defined as the drug concentration that inhibited cell growth by 50%. These values were calculated by regression analysis of the cell survival data. Values are the means ± SD of 3–6 independent experiments performed in triplicate.

* $P < 0.001$ as compared with the IC_{50} of taxol alone.

† $P < 0.05$ as compared with the IC_{50} of taxol alone.

‡ $P < 0.001$ as compared with the IC_{50} of taxol against each of the rodent cell lines.

immunoblotting and northern blotting, respectively. Western blots and immunohistochemistry (data not presented) showed very weak P-glycoprotein positive staining with WR cells (Fig. 1A, lane 3) and a questionable band in the CHO cells (Fig. 1A, lane 4). P-glycoprotein mRNA expression was clearly detectable only in the WR cells by northern blotting (Fig. 1B, lane 3), albeit at very low levels as compared with the positive control. However, no expression of P-glycoprotein mRNA was detected in WS, CHO, NIH3T3, Daudi, K562, 2008, 2008/C13*, 2780 and C70 cells. The presence of MRP mRNA was undetectable by northern blotting in all the cell lines used in this study (data not presented).

RT-PCR assays were performed to assess the presence of MDR1 and MRP transcripts in all the cell lines. A 167 bp MDR1 transcript band was visible only in the P-glycoprotein positive control cells, weakly in the WR, and questionably in the CHO cells (Fig. 2A, lanes 1, 3, and 5, respectively). However, a 230 bp product was consistently amplified in all the human cell lines using the MDR1 specific primers (Fig. 2A, lanes 6–11). The identity of this 230 bp product is unknown at the present time. The primers utilized for amplification of MDR1 transcripts were exon-specific [39]; thus, the 230 bp product could not be due to DNA contamination of the RNA used for reverse transcription. Moreover, control reactions performed in the absence of the AMV reverse transcriptase did not give rise to any amplified product (data not shown). Thus, amplification of the 230 bp product may be the result of employing 35 PCR cycles (a relatively high number), which was required to detect even minimal levels of MDR1 in the WR cells. Basal levels of the amplified MRP product (297 bp) were present in all cell lines (Fig. 2B). However, the rodent cells had lower levels of this transcript when compared with the human cell lines (Fig. 2B, lanes 1–5).

To determine whether transport of taxol played a role in its cytotoxicity, influx and efflux studies using [^{3}H]taxol were initiated. As seen in Fig. 3, the influx of taxol in the rodent

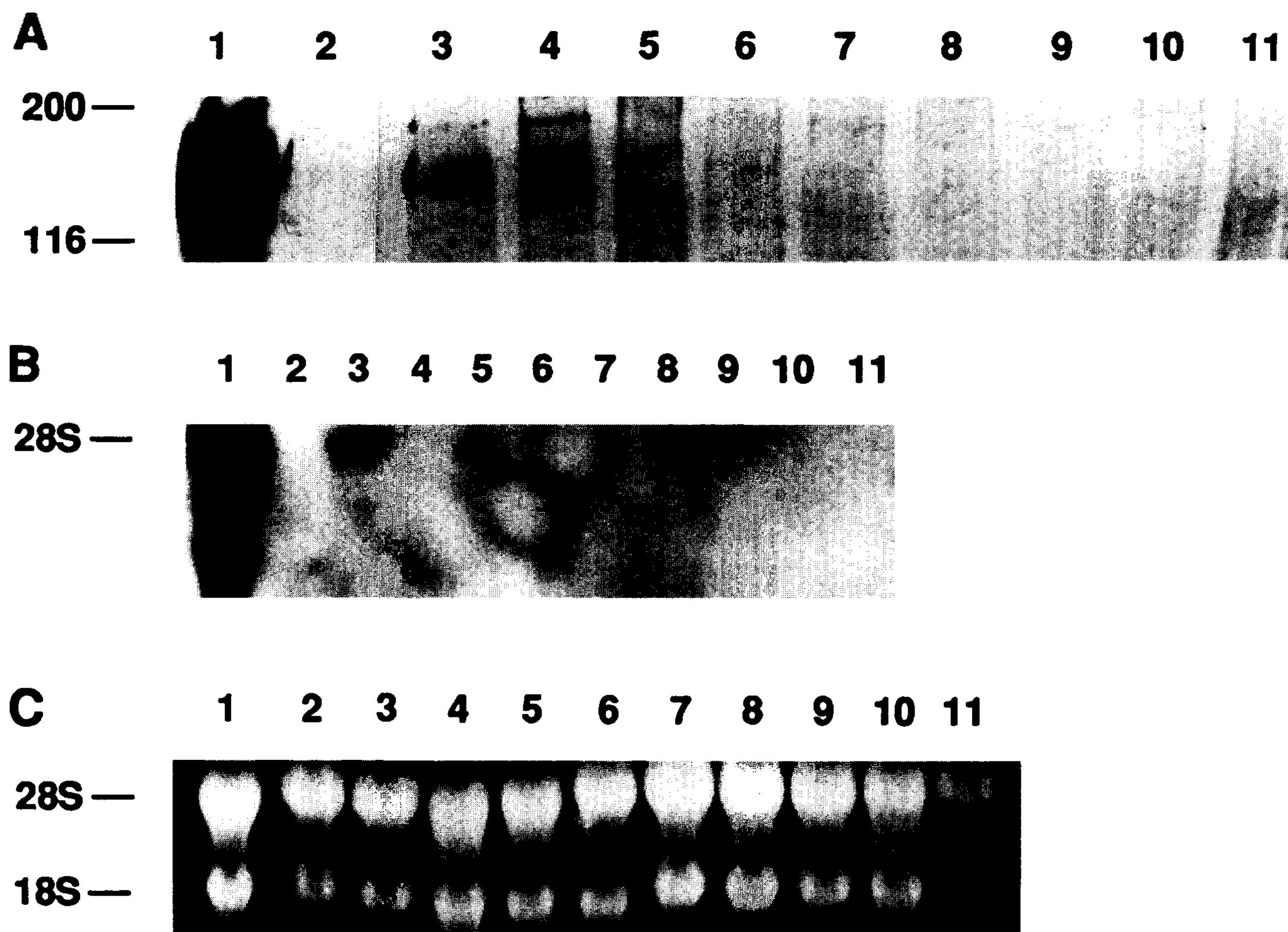

FIG. 1. Immunoblot (A) and northern blot (B) analysis of P-glycoprotein expression in rodent and human cells. The CR1R12 (CHO variant expressing P-glycoprotein) cell line was used as a positive control. Semipurified membrane-enriched fractions (100 μg) from each cell line were subjected to electrophoresis, transferred to Immobilon-P membranes, and immunostained with P-glycoprotein specific antibody. Total cellular RNA was extracted from each cell line, and 30 μg was subjected to electrophoresis, transferred to Nytran Plus membranes, and hybridized with a P-glycoprotein specific probe. (C) Ethidium bromide-stained RNA gel as a control for the amount of RNA loaded in each lane. Lane 1, CR1R12; lane 2, WS; lane 3, WR; lane 4, CHO; lane 5, NIH3T3; lane 6, Daudi; lane 7, K562; lane 8, 2008; lane 9, 2008/C13*; lane 10, 2780; and lane 11, C70. The relative intensity of each band was scanned using an LKB laser densitometric scanner.

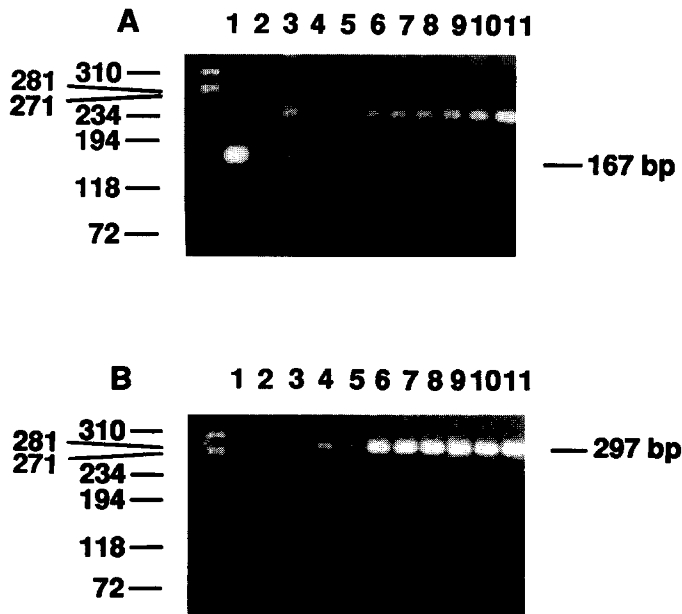

FIG. 2. Relative expression of MDR1 (A) and MRP (B) mRNA as assessed by RT-PCR. Total cellular RNA (1 μg) was reverse transcribed, and the resulting cDNA mixture (0.2 vol.) was amplified using MDR1- and MRP-specific primers for 35 and 30 cycles, respectively, in a total volume of 25 μL. The mixture (10 μL) was then analyzed by agarose gel electrophoresis as described in Materials and Methods. A representative photograph of an ethidium bromide stained gel is shown. Numbers on the left are the size of DNA markers in base pairs. Lane 1, CR1R12; lane 2, WS; lane 3, WR; lane 4, NIH3T3; lane 5, CHO; lane 6, Daudi; lane 7, K562; lane 8, 2008; lane 9, 2008/C13*; lane 10, 2780; and lane 11, C70.

cells ranged from 0.3 to 0.45 nM/mg protein. Furthermore, the efflux of taxol from these rodent cells was also very rapid with >75% taxol being released from the cells in 10–30 min. In contrast, the intracellular levels of taxol in the human cells ranged from 1.2 to 1.7 nM/mg protein, and these cells also retained more than 50% taxol after 4 hr. The 2008 cells were an exception; they retained <20% of taxol following 4 hr of incubation (Fig. 3). Pretreatment of the rodent cells with 10 μM verapamil led to an increased influx of taxol (between 2- and 4-fold) at the end of 4 hr, while no significant effect of verapamil pretreatment on taxol uptake was observed with the human cells (Table 2).

The specific binding of [^{3}H]taxol in the rodent and human cell lines was calculated in the presence of excess unlabeled taxol, and the results are presented in Table 3. No specific binding of [^{3}H]taxol was detectable in the Walker rat lymphoma cells. The specific binding was 0.092 and 0.158 nM/mg protein in the CHO and NIH3T3 cells, respectively, and the specific binding of labeled taxol in all the human tumor cells was 4- to 9-fold greater than in either the CHO or NIH3T3 cells.

Polymerized tubulins are the intracellular targets of taxol [8–10]. Thus, α- and β-tubulin protein levels were determined in all the cell lines following immunostaining with specific antibodies and their RNA levels by northern blotting. Although the α-tubulin protein levels appear more variable than β-tubulin (Fig. 4, A and B, respectively), no significant differences in the intracellular concentrations of the total α- and β-tubulin proteins were apparent after western blot analyses of the whole cell homogenate. Furthermore, northern blot analysis revealed that, the levels of α-tubulin mRNA were also variable (Fig. 4C), while levels of the mRNA for β-tubulin were similar in all the cell lines studied (Fig. 4D).

Taxol binds to the β-tubulin subunit in the microtubule polymer [11–13]. Thus, the basal levels of polymerized and soluble tubulins were determined in all the cell lines. Western blot analysis of the separated proteins revealed that cells growing in suspension culture, i.e. WS, WR, Daudi, and K562, contained the lowest amount of polymerized tubulins (data not shown), but no correlation was found between taxol chemosensitivity and the amount of polymerized tubulin.

To assess whether the decreased specific binding of [^{3}H]taxol in the rodent cells when compared with the human tumor cells was due to a mutation in the taxol binding site, microtubule proteins from WS, CHO, and 2008/C13* cells were purified by the two cycles of assembly and disassembly. As judged by SDS-PAGE, the microtubule preparation from WS, CHO, and 2008/C13* consisted mainly of the tubulins (Fig. 5A), with minor impurities, probably the microtubule-associated proteins. *In vitro* binding was performed utilizing 0.5 mg/mL of the microtubule proteins with different concentrations of labeled taxol. As seen in Fig. 5B, the *in vitro* binding of [^{3}H]taxol to microtubule preparations from all three cell lines was similar. This binding was inhibited by the addition of excess (100-fold) unlabeled taxol, confirming the specificity of the binding reaction.

DISCUSSION

In the present study, we have shown that rodent cells were between 15- and 100-fold less sensitive to taxol than all the human tumor cells, except for the human ovarian carcinoma (2008) cells, which had intermediate sensitivity to this drug. Transport studies revealed that the rodent cells, following 4 hr of incubation, had accumulated approximately 3- to 6-fold less taxol than the human cells. Moreover, a rapid efflux of taxol was observed in the rodent cells, wherein >90% of the taxol was transported out of the cells within 4 hr. In contrast, 50% or more of the intracellular taxol was retained in human tumor cells at the end of 4 hr. Due to the hydrophobic nature of taxol, it has been suggested that taxol is transported across the membrane by passive diffusion [9], but such a mechanism is unlikely to explain the observed differences in taxol accumulation and efflux between the rodent and human cells. Furthermore, these differences are not limited to taxol. Recent reports have shown that the toxicity and transport of antimitotic agents including colchicine, colcemid, maytansine, nocodazole, taxol, and vinblastine were markedly different when these parameters were measured in human cervical carcinoma (HeLa) cells and compared with CHO cells [28, 29].

Pretreatment of rodent cells with non-toxic concentrations of verapamil sensitized them to taxol cytotoxicity and in-

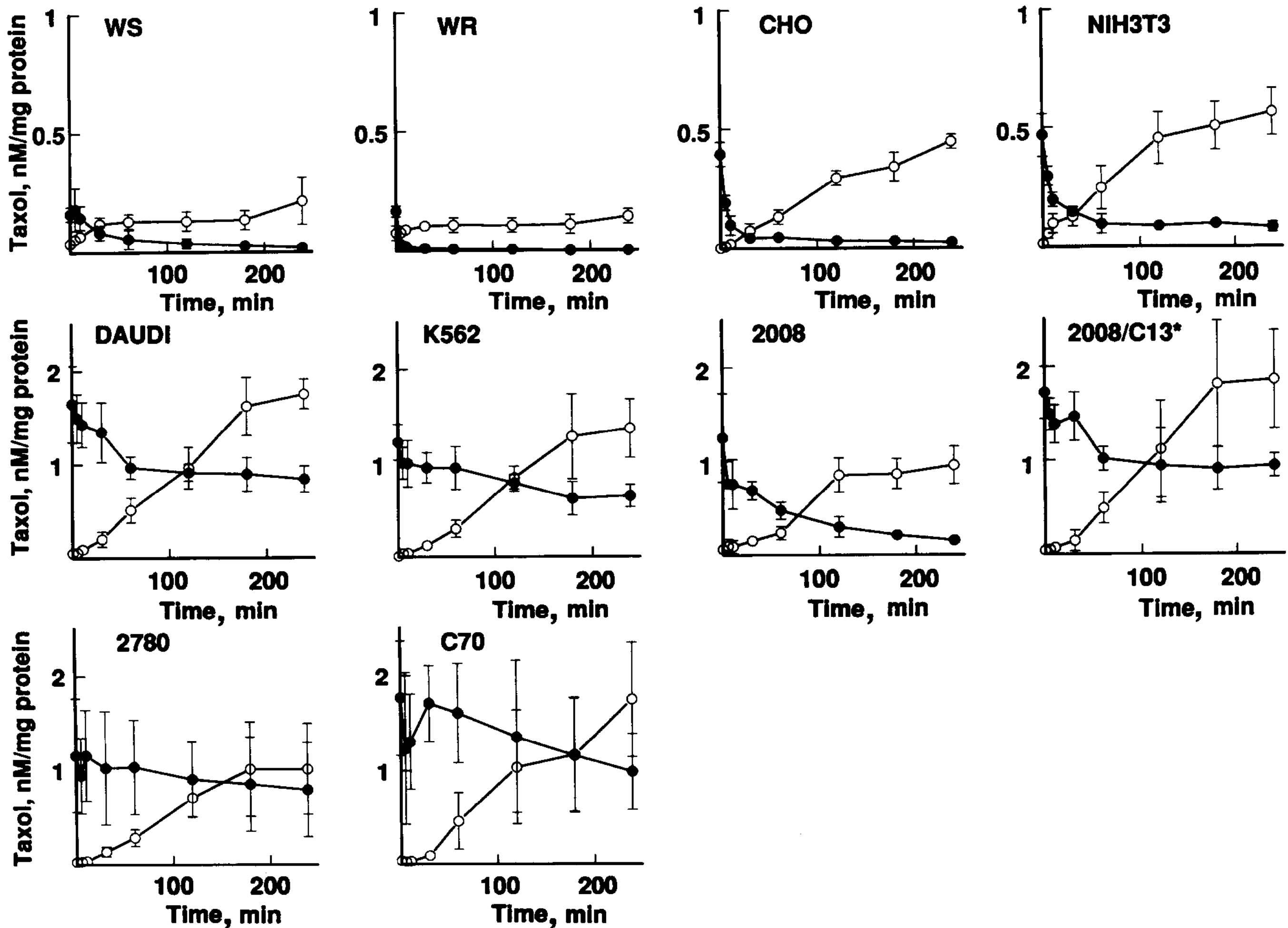

FIG. 3. Influx (○) and efflux (●) of [^{3}H]taxol in rodent and human cells. Influx was determined after incubating cells with 5.16 nM [^{3}H]taxol for the indicated time periods. After each incubation period, the cells were washed thoroughly with chilled phosphate-buffered saline and lysed in 0.1 N NaOH overnight. Efflux of [^{3}H]taxol was determined after loading the cells by exposure to 5.16 nM [^{3}H]taxol for 4 hr. Cells were then washed with chilled phosphate-buffered saline and incubated in fresh medium for the indicated time periods. After each incubation period, the cells were treated as described above. Radioactivity was determined in a Beckman liquid scintillation counter. Each point is the mean ± SD of 3 separate experiments, each performed in triplicate.

creased the intracellular accumulation of taxol by a significantly greater extent to that observed with the human tumor cells. The utility of verapamil as a drug response modulator in resistant tumor cells, especially in MDR tumor cells, is well documented, and it has been postulated that verapamil inhibits the energy-dependent efflux of natural product drugs by competing for the drug-binding site on the P-glycoprotein [20]. In the present study, P-glycoprotein expression was undetectable in all the cell lines, except WR (a cisplatin-resistant subline of the parental WS cells), in which a low level of P-glycoprotein expression was observed. RT-PCR analysis (a much more sensitive detection technique) also showed that the P-glycoprotein transcript was only detectable in the WR cells, although 35 cycles of PCR, capable of detecting extremely low levels of MDR1 transcript [39], were necessary before the transcript was detected in these cells. These results clearly show that P-glycoprotein expression is essentially absent in all the cell lines utilized in this study, except WR. Thus, the 15- to 100-fold resistance (as compared with human cells), and the marked sensitization to taxol induced by pretreatment with verapamil of the rodent cells cannot simply be explained by expression of the P-glycoprotein in the rodent cells. However, P-glycoprotein cannot be ruled out completely since little correlation exist between P-glycoprotein efflux activity and the amount of P-glycoprotein [20].

Recently, overexpression of MRP has been demonstrated in certain P-glycoprotein negative, MDR tumor cells [31, 42–46]. Similar to the P-glycoprotein, MRP is present in the cell membrane of drug-resistant cells and functions as a drug efflux pump [31, 42–46]. However, unlike P-glycoprotein, it is not known whether taxol is a substrate for MRP. In the present study, MRP expression, as assessed by northern blotting, was undetectable in both the rodent and the human cells (data not presented). RT-PCR analysis utilizing human MRP specific primers revealed that the basal levels of MRP transcript were greater in the human cells than in the rodent cells. These

TABLE 2. Effect of a 4-hr pretreatment with 10 μM verapamil on the accumulation of taxol

Cell lines	Taxol accumulation (nM/mg protein)		Fold-increase
	Taxol alone	Verapamil + taxol	
Rodent cells			
WS	0.24 ± 0.06	0.51 ± 0.10	2.1
WR	0.14 ± 0.03	0.58 ± 0.09	4.1
CHO	0.48 ± 0.04	0.95 ± 0.25	2.0
NIH3T3	0.57 ± 0.10	0.94 ± 0.30	1.6
Human cells			
Daudi	1.50 ± 0.20	1.60 ± 0.30	1.0
K562	1.20 ± 0.20	1.40 ± 0.40	1.2
2008	1.07 ± 0.20	1.50 ± 0.40	1.4
2008/C13*	1.55 ± 0.50	1.70 ± 0.30	1.1
2780	1.10 ± 0.35	1.30 ± 0.40	1.2
C70	1.65 ± 0.45	1.75 ± 0.60	1.05

Tumor cells were seeded (2×10^6 cells/well) in a 6-well plate and treated with 10 μM verapamil for 4 hr prior to exposure to 5.16 nM [^{3}H]taxol for 4 hr. At the end of the exposure period, the cells were washed five times with chilled phosphate-buffered saline and lysed with 0.1 N NaOH overnight. Radioactivity was determined in a Beckman liquid scintillation counter. Values are the means ± SD of 3 independent experiments performed in duplicate.

results indicate that MRP is unlikely to play an important role in the relative resistance of the rodent cells to taxol because if taxol were a good substrate for MRP, human cells should exhibit increased resistance to the drug as compared with the rodent cells, when, in fact, the opposite is true.

The intracellular levels of tubulins appear to be altered in some cell lines resistant to taxol [24, 25]. Furthermore, alterations in the α- and β-tubulin proteins have also been reported in taxol-resistant CHO cells [24]. In the present study, the intracellular concentrations of the α- and β-tubulins were found to be similar in the rodent and human cells. However, northern blotting analysis revealed that while the levels of the β-tubulin mRNA were similar in rodent and human cells, the levels of α-tubulin mRNA were lower in the rodent than in the human cells. Taxol binds primarily to the N-terminal region of β-tubulin in the microtubule polymer [13], and Minotti *et al.* [25] reported that resistance to antimitotic drugs in some clones of CHO cells could be correlated to changes in the intracellular content of polymerized tubulin. Analysis after extraction of microtubule proteins from the rodent and human cells revealed that cells grown as a suspension culture (WS, WR, Daudi, and K562) possess the lowest levels of polymerized tubulin when compared with cells grown as a monolayer (NIH3T3, CHO, 2008, 2008/C13*, 2780, and C70). However, no correlation between the intracellular amounts of polymerized tubulins and taxol sensitivity was observed.

Structural and functional alterations of tubulins have been shown to alter their drug-binding affinity [22–24, 26, 27]. In

TABLE 3. Specific Binding of [^{3}H]Taxol

Cell lines	Binding (nM/mg protein)	
	Total	Specific*
Rodent cells		
WS	0.051 ± 0.005	0
WR	0.047 ± 0.004	0
CHO	0.260 ± 0.03	0.092
NIH3T3	0.426 ± 0.09	0.158
Human cells		
Daudi	1.054 ± 0.21	0.752†
K562	0.984 ± 0.16	0.598†
2008	0.787 ± 0.04	0.574†
2008/C13*	1.04 ± 0.07	0.77†
2780	0.780 ± 0.05	0.564†
C70	0.857 ± 0.1	0.638†

Total binding in the presence of 5.16 nM [^{3}H]taxol was determined at the end of 2 hr. Non-specific binding was determined in the presence of 5.16 nM [^{3}H]taxol and 100-fold excess of unlabeled taxol, at the end of 2 hr. Values are the means ± SD of 3 separate experiments performed in duplicate.

* Non-specific binding was in the presence of 100-fold excess of unlabeled taxol. Specific binding was determined by subtracting the non-specific binding from the total binding.

† $P < 0.001$ as compared with specific binding observed in each of the rodent cell lines.

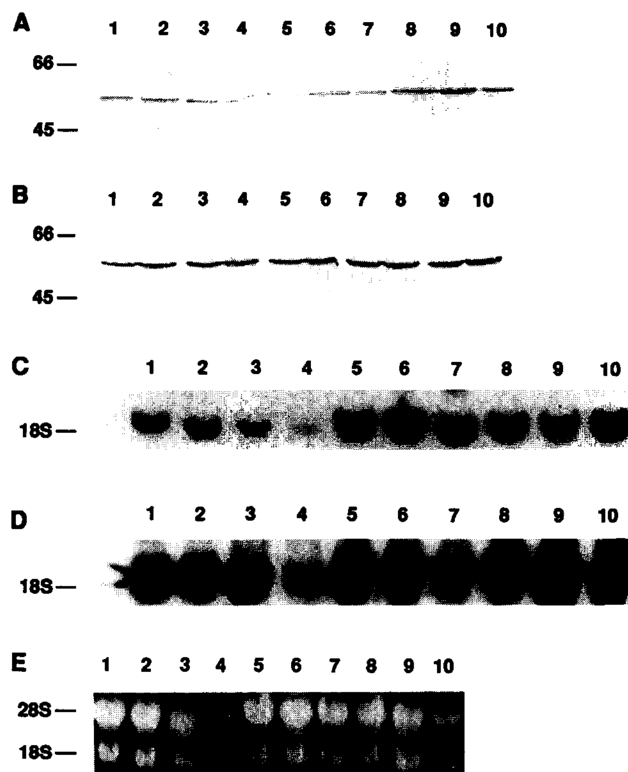

FIG. 4. Immunoblot (A, B) and northern blot (C, D) analysis of α- and β-tubulin expression in rodent and human cells. Protein extracts equal to 2×10^5 cells were subjected to electrophoresis, transferred to Immobilon-P membranes, and immunostained with α-tubulin (A) and β-tubulin (B) specific antibodies. Total cellular RNA was extracted from each cell line, and 30 μg was subjected to electrophoresis, transferred to Nytran Plus membranes, and hybridized with α-tubulin (C) and β-tubulin (D) specific probes. (E) Ethidium bromide-stained RNA gel as a control for the amount of RNA loaded in each lane. Lane 1, WS; lane 2, WR; lane 3, CHO; lane 4, NIH3T3; lane 5, Daudi; lane 6, K562; lane 7, 2008; lane 8, 2008/C13*; lane 9, 2780; and lane 10, C70. The relative intensity of each band was scanned using an LKB laser densitometric scanner.

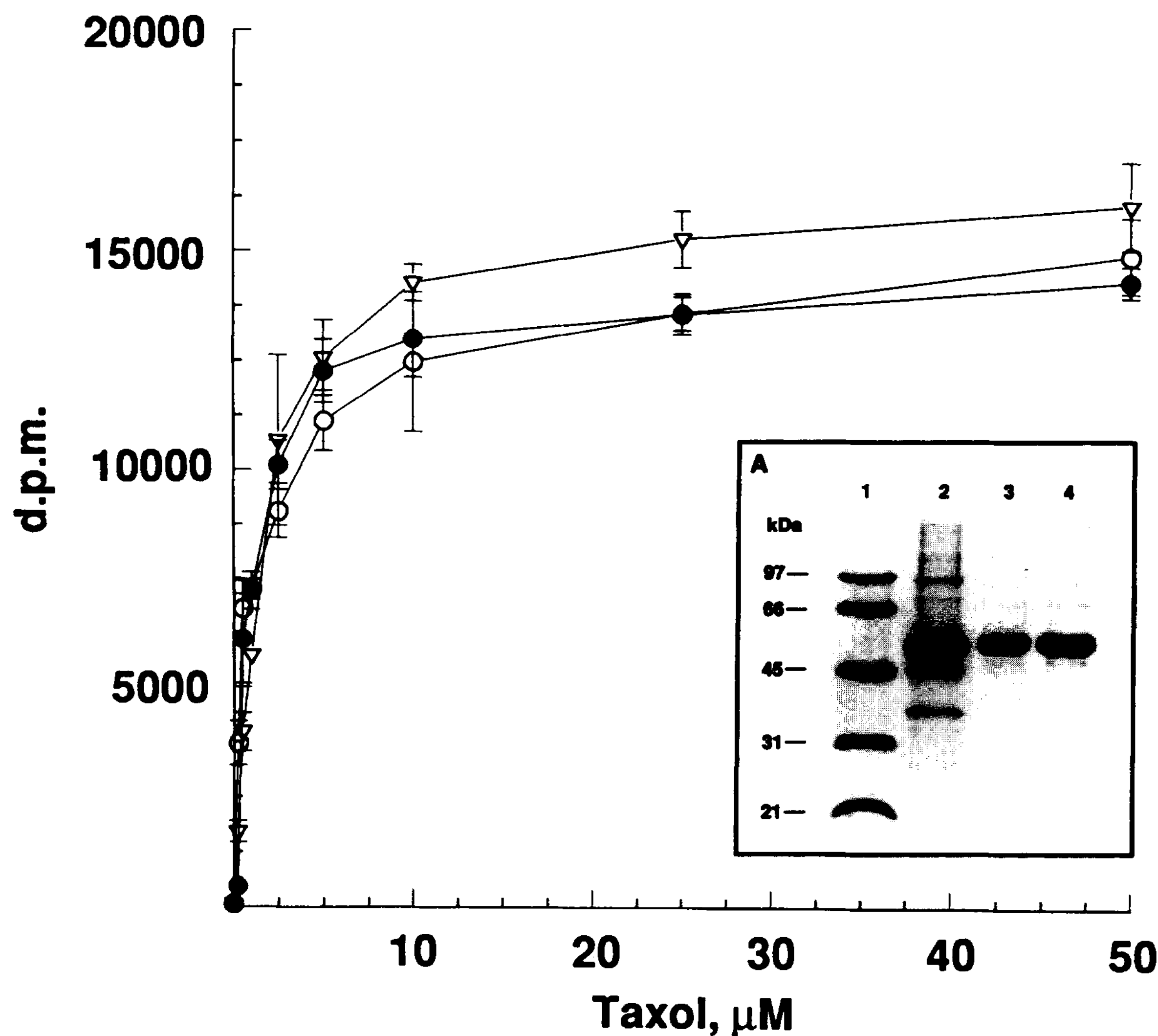

FIG. 5. (A) Coomassie blue-stained SDS-PAGE of purified microtubule proteins (5 µg/lane) from WS (lane 2), CHO (lane 3) and 2008/C13* (lane 4) cells. Molecular weight markers are shown in lane 1. Microtubule proteins were purified as described in Materials and Methods. (B) *In vitro* binding of [^{3}H]taxol with purified microtubule proteins from WS (○), CHO (●), and 2008/C13* (▽) cells. [^{3}H]Taxol (0.25 to 50 µM) was added to microtubule protein (0.5 mg/mL) in assembly buffer and incubated for 45 min at 37°. Thereafter, one-half of the reaction mixture was layered onto GF/C glass fiber filter discs and washed with phosphate-buffered saline to remove unbound taxol. The filter paper disc was allowed to dry overnight, and then the radioactivity was measured. Each point is the mean ± SD of 2 separate experiments, each performed in triplicate.

the present study, we observed a reduced intracellular binding of taxol in the rodent as compared with the human tumor cells. It was therefore of interest to analyze whether the reduced specific binding in the rodent cells was due to a decreased influx/increased efflux of taxol or due to an alteration in the drug binding affinity of the microtubule proteins from these rodent cells. Thus, microtubule proteins were semi-purified from cell lines exhibiting low (CHO), intermediate (WS) and high (2008/C13*) chemosensitivity to taxol. No significant difference in the binding of taxol to microtubule protein preparations from these cells was detected. Thus, observations with the mitotic spindle poisons (colcemid and vincristine) showing that the altered binding affinities of these drugs to tubulin can be correlated to their cytotoxicity [22, 23] cannot be extended to explain the relative taxol resistance of these rodent cell lines.

In summary, rodent tumor cells are less sensitive than human tumor cells to taxol. This is associated with a significantly reduced intracellular accumulation and binding of taxol in rodent cells compared with the human tumor cells. Reduced intracellular drug levels in rodent cells cannot be correlated with the expression of either P-glycoprotein or MRP. In fact, the human cells have greater levels of MRP as judged by RT-PCR, suggesting that taxol is either not a good substrate for MRP or that MRP is not an important participant in the transport of taxol. Furthermore, no difference was observed in the binding of taxol to tubulins between rodent and human tumor cells; thus, alterations in tubulin structure cannot explain the taxol resistance of these rodent tumor cells. Taken together, the evidence presented in the current study prompts us to propose the presence of a hitherto undefined transport system for taxol and probably for other drugs in these rodent cell lines. This alternative transport system may also explain the 10-fold resistance to taxol, lower intracellular accumulation, and rapid efflux of taxol exhibited by the human ovarian carcinoma (2008) cells compared with the taxol sensitivity of the 2008/C13* (cisplatin-resistant variant of 2008) cells, and the other human tumor cells. Similar differences in taxol sensitivity and transport between 2008 and 2008/C13* cells have been reported previously [47, 48]. The observation that a novel phosphoglycoprotein, distinct from the P-glycoprotein is overexpressed in a taxol-resistant murine macrophage-like cell line [30] lends credence to this notion. Our present studies are being extended to attempt to define at the molecular level the "taxol transport system" in rodent cells, employing photolabeled derivatives of taxol.

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