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Taxol Analogues Exhibit Differential Effects on Photoaffinity Labeling of β -Tubulin and the Multidrug Resistance Associated P-Glycoprotein

Chia-Ping Huang Yang^{*,†,#}, Changwei Wang[‡], Iwao Ojima[‡], and Susan Band Horwitz[†]

[†]Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, United States

[#]Department of Obstetrics and Gynecology and Women's Health, Division of Gynecologic Oncology, Albert Einstein College of Medicine, Bronx, New York 10461, United States

[‡]Department of Chemistry and Institute of Chemical Biology and Drug Discovery, State University of New York at Stony Brook, Stony Brook, New York 11794, United States

Abstract

Several next-generation taxanes have been reported to possess high potency against Taxol-resistant cancer cell lines overexpressing β III-tubulin and/or P-glycoprotein (P-gp), both of which are involved in drug resistance. Using a photoaffinity Taxol analogue, 2-(*m*-azidobenzoyl)-taxol, two potent next-generation taxanes, SB-T-1214 and SB-CST-10202, exhibited distinct inhibitory effects on photolabeling of β -tubulin from different eukaryotic sources that differ in β -tubulin isotype composition. They also specifically inhibited photolabeling of P-gp, and the inhibitory effect correlated well with the steady-state accumulation of [³H]vinblastine in a multidrug resistant (MDR) cell line, SKVLB1. Several microtubule-stabilizing agents (MSAs)-resistant cell lines from the human ovarian cancer cell line Hey were isolated, and their *MDR1* and β III-tubulin levels determined. Distinct potencies of the two taxanes against different MSA-resistant cells expressing unique levels of *MDR1* and β III-tubulin were found. Cytotoxicity assays, done in the presence of verapamil, indicated that SB-T-1214 is a substrate, although not as good as Taxol, for P-gp. The mechanisms involved in drug resistance are multifactorial, and the effectiveness of new Taxol analogues depends on the interaction between the drugs and all possible targets; in this case the two major cellular targets are β -tubulin and P-gp.

Microtubules represent an important target for antitumor drugs of natural product origin, such as the taxanes and the epothilones. Our laboratory has studied extensively the microtubule-stabilizing agent (MSA), Taxol (paclitaxel), that has been approved for treatment of a variety of malignancies, including ovarian, breast, and lung carcinomas. Ixabepilone, another MSA, has been approved for treating metastatic breast cancer. Taxol

*Corresponding Author Tel: (718)430-2361. chia-ping.h.yang@einstein.yu.edu.

Notes

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DEDICATION

The other coauthors of this article dedicate it to Dr. Susan Band Horwitz, of Albert Einstein College of Medicine, Bronx, NY, for her pioneering work on bioactive natural products.

abolishes normal microtubule function by stabilizing the polymer, thereby altering the dynamicity of microtubules which results in inhibition of cell division plus interfering with events in interphase cells such as microtubule trafficking.¹

Microtubules are heterodimers composed of α - and β -tubulin. Early photoaffinity labeling studies, using a variety of Taxol analogues,² and electron crystallography research done by Nogales et al.³ indicated that β -tubulin contains the binding site for Taxol. There are eight α - and eight β -tubulin isotypes present in distinct quantities in different mammalian cells.⁴ Differential expression of tubulin isotypes and altered post-translational modifications (PTMs) of tubulin have been observed in cancers.⁵ In several cancers and in some drug-resistant cancer cell lines, overexpression of β III-tubulin has been associated with resistance to Taxol and other classes of antitumor drugs.⁶ Increased expression of β III-tubulin also was seen in several MSA-resistant cell lines.⁷

The molecular mechanisms that underlie MSA-mediated drug resistance are complex and multifaceted. P-gp, the gene product of *MDR1*, is overexpressed in MDR cells and some tumors. It acts as a drug-efflux pump to maintain low concentrations of hydrophobic chemotherapeutic drugs in the cell.⁸ In addition to binding to microtubules, the taxanes are known to bind to P-gp in cells. The relative binding affinities of the taxanes to both microtubules and P-gp will influence the therapeutic efficacy of the drugs. Since β III-tubulin is overexpressed in some drug-resistant tumors, there have been attempts to design drugs targeting this isotype, particularly its M-loop, which is known to be involved in drug binding.^{2c,3} It has been reported that the next-generation taxanes are active against taxane-resistant cell lines that overexpress P-gp and/or β III-tubulin.⁹ In this study we examined the effect of two potent next-generation taxanes, SB-T-1214¹⁰ and SB-CST-10202^{9b} (Figure 1), on the binding of a photoaffinity Taxol analogue, 2-(*m*-azidobenzoyl)taxol (2-m-AzTax),¹¹ to P-gp and β -tubulin, and on the growth of the ovarian cancer cell line Hey and its MSA-resistant daughter cell lines that overexpress different levels of P-gp and β III-tubulin.

RESULTS AND DISCUSSION

Since the taxanes bind to both tubulin and P-gp, the relative binding affinity of the taxanes to these two cellular targets may influence the effectiveness of the taxanes in resistant cells. To determine the effects of SB-T-1214 and SB-CST-10202 on binding of Taxol to P-gp and β -tubulin, a tritium-labeled Taxol analogue, 2-(*m*-azidobenzoyl)taxol (2-m-AzTax), was used. We have demonstrated previously that 2-m-AzTax photolabels a peptide (amino acids 217–231) in β -tubulin.^{2a} To study the relative binding affinities of SB-T-1214 and SB-CST-10202, tubulins from bovine brain (BBT) and chicken erythrocytes (CET) were specifically photolabeled with [³H]2-m-AzTax in the presence and absence of either Taxol, Taxotere, SB-T-1214, or SB-CST-10202. β -Tubulin isotype content in BBT and CET is different; BBT contains β I-, β II, β III, and β IV-tubulins, whereas CET has only β VI. Taxol had a minimal inhibitory effect (~9%) on BBT, but a strong inhibitory effect (98%) on CET. Taxotere had a stronger inhibitory effect than Taxol on photolabeling of BBT (33% and 98% for BBT and CET, respectively). The inhibitory effects elicited by the two next-generation taxanes on photolabeling were distinct for β -tubulins from these two sources. SB-T-1214 and SB-CST-10202 inhibited BBT photolabeling by 38% and 32%, respectively.

Interestingly, these two drugs caused a 30% and only 6% inhibition, respectively, on photolabeling of CET that only contains β VI-tubulin (Figure 2). These results indicated that these two next-generation taxanes have a binding affinity similar to that of Taxotere for BBT, but are very different for CET, suggesting that their binding to β -tubulin is isotype specific.

These results suggest that the two next-generation drugs would have distinct effects on different cancer cells, depending on the isotype profiles of tubulin expressed in the cells. Since there is no marked inhibition of photolabeling by the two taxanes with CET that expresses only β VI-tubulin, it is suggested that SB-T-1214 and SB-CST-10202 would not be very effective against β VI-tubulin-overexpressing cells, such as leukocytes and platelets.

To study photolabeling of P-gp, plasma membranes from an MDR cell line, SKVLB1,¹² that expresses very high levels of P-gp were prepared (Figure 3A). SB-T-1214 and SB-CST-10202 inhibited photolabeling of P-gp markedly (>80% inhibition), indicating that these two taxanes bind to P-gp (Figure 3B). They also caused an increase in steady-state [³H]vinblastine accumulation in the MDR cell line SIKVLB1,¹² compared to the drug-sensitive SKOV3 cells (Figure 3C), which is due to the binding of the competing drugs to P-gp, thereby preventing [³H]vinblastine from being pumped out of the resistant cells. A good correlation between the effects of Taxol, Taxotere, and these two next-generation taxanes on inhibition of photolabeling and [³H]vinblastine accumulation was obtained ($R^2 = 0.893$) (Figure 3D). For example, steady-state accumulation studies indicated that the [³H]vinblastine level in SKVLB1 cells treated with SB-T-1214 is much higher than that in control cells. Taxotere inhibited P-gp photolabeling by 55% (compared to 89% for SB-T-1214), and the steady-state [³H]vinblastine level for taxotere-treated SKVLB1 cells is much lower than that for SB-T-1214, suggesting that Taxotere is less effective than SB-T-1214 in preventing vinblastine from being pumped out of SKVLB1 cells.

Three MSA-resistant cell lines were derived from the human ovarian cancer cell line Hey: Taxol-resistant Hey.Tx100, ixabepilone-resistant Hey.Ixab80, and epothilone B-resistant Hey.EpoB8.⁷ They are approximately 90-fold resistant to Taxol, 7-fold resistant to ixabepilone, and 15-fold resistant to EpoB, respectively. Hey.Tx100 expresses high levels of *MDR1*, Hey.Ixab80 expresses moderate amounts of *MDR1*, and Hey.EpoB8 essentially does not express *MDR1*, compared to the sensitive Hey cells (Figure 4A). Expression of β III-tubulin in Hey.Tx100, Hey.Ixab80, and Hey.EpoB8 cells was increased by 2.9-, 8.7-, and 4.5-fold, respectively (Figure 4B). We examined the effect of two potent next-generation taxanes, SB-T-1214 and SB-CST-10202, on growth of the sensitive and its MSA-resistant daughter cell lines. We found that in Hey.Tx100 and Hey.Ixab80, both of which express increased levels of P-gp, SB-T-1214 was extremely potent in inhibiting cell growth and was ~6–9-fold more potent than SB-CST-10202 against these drug-resistant cells. However, in Hey.EpoB8 cells that expressed increased levels of β III-tubulin, but did not express *MDR1*, SB-T-1214 and SB-CST-10202 did not increase the drug sensitivity of these resistant cells (Table 1 and Figure 4C). These results indicate that these two taxanes, particularly SB-T-1214, are effective primarily against P-gp-overexpressing cells. It is noted that tubulins belong to a multifunctional cellular protein family comprising several isotypes. Binding of drugs to different isotypes may induce diverse downstream cellular events. Further study is

needed to determine whether MSA-induced inhibition of Taxol analogue binding to β -tubulin correlates with the ability of MSA-mediated inhibition of cell proliferation.

A highly vinblastine-resistant human ovarian cancer cell line, SKVLB1, which was derived from the parental cell line SKOV3 and is approximately 2000-fold resistant to vinblastine, expresses approximately 100-fold more P-gp than the drug-sensitive SKOV3 cells¹² (Figure 5A), but has a reduced level of β III-tubulin (approximately 40% reduction, Figure 5B). Both SB-T-1214 and SB-CST-10202 were markedly potent against this resistant cell line (Table 2 and Figure 5C).

To determine whether SB-T-1214 is a substrate for P-gp, cytotoxicity assays were done in the absence and presence of verapamil (Figure 6A). While verapamil had no effect on the growth of drug-sensitive SKOV3 cells in the presence of increasing concentrations of SB-T-1214 (Figure 6A), 2 μ M verapamil decreased the IC₅₀ of SB-T-1214 in SKVLB1 cells by approximately 4-fold (Table 2 and Figure 6B and C), strongly suggesting that SB-T-1214 is a substrate for P-gp. When compared with Taxol, which is known as an excellent substrate for P-gp, 2 μ M verapamil decreased the IC₅₀ of Taxol by approximately 6-fold. Therefore, although SB-T-1214 is a substrate for P-gp, it is not as good as Taxol (Table 3).

Drug resistance remains a major obstacle for oncologists treating cancer with MSAs. The complex and multifaceted mechanisms that underlie resistance from MSAs are not completely understood, and scientists continue to search for targets or biomarkers that are involved in MSA-mediated drug resistance. P-gp and/or β III-tubulin are known targets for MSAs, and researchers have tried to synthesize Taxol analogues that are potent against Taxol-mediated drug resistance. Since the M-loop is involved in the binding of drugs to β -tubulin,³ drugs have been designed to target this domain of β III-tubulin. For example, IDN5390, a seco-taxane in which the six-membered C ring of the baccatin structure is open, has been demonstrated to target β III-tubulin.^{9a} Other C-seco-taxoids, such as SB-CST-10202, possess high potency against β III-tubulin-overexpressing Taxol-resistant A2780 cell lines selected in the presence of a P-gp blocker, cyclosporine.^{9b} In addition, several taxanes with chemical modifications at C10 and C13 positions have high potency for inducing tubulin assembly, compared with other taxanes. These compounds are active against all types of Taxol-resistant cell lines, including cells that are P-gp overexpressing, are β III-tubulin overexpressing, or contain tubulin binding site mutations.^{9c}

In this study we further analyzed the influence of two next-generation taxanes on binding to P-gp and β III-tubulin and examined the effectiveness of these two Taxol analogues on the growth of MSA-resistant ovarian cancer cells. We found that (i) both SB-T-1214 and SB-CST-10202 specifically bind to P-gp, and their inhibitory effects on P-gp photolabeling correlate well with an increase in steady-state vinblastine accumulation; (ii) these two taxanes exhibit distinct inhibitory effects on photolabeling of β -tubulin from different eukaryotic sources, suggesting the importance of knowing the β -tubulin isotype content in a tumor. Since drug binding to tubulin and P-gp are the primary events that occur in the cell following drug administration, our data on taxane binding affinity to these two targets may help in understanding the effectiveness of next-generation taxanes. In addition, we have

demonstrated that these two taxanes, particularly SB-T-1214, are very potent against MSA-resistant cells, and they preferentially influence the cells overexpressing P-gp.

We have reported¹³ that, in addition to the M-loop, the leucine cluster region of β III-tubulin, which is known to be important in the interaction of the drug with the micro-tubule,^{2c, 14} contains a unique residue, alanine, at 218, compared to other isotypes that have threonine at this position. Using protein structure models for the β I-tubulin monomer (containing Thr218) with Taxol in the binding pocket, it has been demonstrated that no contacts (such as hydrogen bond) between T218 and Taxol were found. Therefore, the lower binding affinity of β III-tubulin cannot be ascribed to loss of direct interaction between Taxol and residue 218. Instead, after running molecular dynamic simulations, it was found that the frequency of Taxol-accommodating conformations decreased significantly in the T218A variant (β III-tubulin), compared with other β -tubulin isotypes. Both photolabeling with 2-m-AzTax and molecular dynamic simulations studies have indicated that a difference in residue 218 in β III-tubulin may be responsible for inhibition of drug binding to this isotype.¹³ The differential binding of the Taxol analogue to β III-tubulin may influence downstream cellular events, and therefore it may be advantageous to design drugs that target the leucine cluster domain of β III-tubulin containing the T218A residue in addition to targeting the M-loop.

EXPERIMENTAL SECTION

Drugs and Tubulin.

Taxol was obtained from the Drug Development Branch, National Cancer Institute. Docetaxel (Taxotere) was obtained from Sanofi. SB-T-1214 and SB-CST-10202 were synthesized in-house using the previously reported methods.^{9b,c} [³H]2-m-AzTax was provided by GlaxoSmithKline, and [³H]vinblastine was purchased from PerkinElmer. Bovine brain tubulin was purchased from Cytoskeleton, Inc. Chicken erythrocyte tubulin was isolated from the marginal bands of chicken erythrocytes as previously described.¹⁵ Monoclonal anti- β III-tubulin antibody was purchased from Covance.

Cell Culture.

Hey cells was obtained from Dr. Gil Mor, Yale Medical School, SKVLB1 cells from Dr. Victor Ling, Ontario Cancer Institute, and SKOV3 from the American Type Culture Collection. Low-passage-number cells were used for all experiments. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum. Resistant cell lines were isolated,⁷ and Hey.Tx100, Hey.Ixab80, Hey.EpoB8, and SKVLB1 were maintained in 100 nM Taxol, 80 nM ixabepilone, 8 nM epothilone B, and 1 μ M vinblastine, respectively.

Photoaffinity Labeling of Tubulin and Drug Competition Assays.

Soluble tubulin (4 μ M) in MEM buffer (0.1 M MES, 1 mM EGTA, 1 mM MgCl₂ pH 6.7) containing 3 M glycerol and 1 mM GTP was incubated with 5 μ M [³H]2-m-AzTax (specific activity: 5.5 Ci/mmol) in the absence or presence of a 4-fold molar excess of competing taxanes (20 μ M) at 37 °C for 30 min.^{2a} The samples were irradiated for 10 min at 4 °C with a UV lamp (254 nm) at a distance of 6 cm. The entire sample containing photolabeled

microtubules was analyzed by SDS-PAGE on 9% gels, using a cathode buffer that was titrated to pH 9, and detected by fluorography.

Measurement of Steady-State Drug Accumulation in Intact Cells.

A total of $(3-4) \times 10^5$ cells were plated in each well of 12-well culture dishes and maintained in drug-free, serum-free RPMI medium for 2 h. The medium was aspirated, and fresh medium containing [^3H]vinblastine ($1 \mu\text{M}$, specific activity = 24 mCi/mmol) plus and minus Taxol analogues was added. Following 1 h of incubation at 37 °C, the cells were washed three times with cold phosphate-buffered saline. Cells were then lysed in 1 N NaOH at room temperature for 16 h. Total protein was determined, and total radioactivity measured by liquid scintillation counting.

Determination of IC₅₀ Values.

One thousand to 2000 cells were added to each well of a 96-well plate. Increasing concentrations of the indicated drugs were added 18 h after plating. The IC₅₀, the concentration of drug that inhibits cell growth by 50%, was determined after 72–120 h of incubation at 37 °C using the sulforhodamine B method.¹⁶ Cell lines were fixed and stained at different times to take into account their growth rates.

Western Blot Analysis.

Denatured cell lysates were prepared as described.¹⁷ Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti- β III-tubulin antibody.

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Figure 1.
Structures of Taxol, Taxotere, SB-T-1214, and SB-CST-10202.

Figure 2.

Both SB-T-1214 and SB-CST-10202 exhibit distinct effects on 2-m-AzTax photoaffinity labeling of β -tubulin from bovine brain (BBT) and chicken erythrocytes (CET). (A) BBT or CET ($4 \mu\text{M}$) was incubated with $5 \mu\text{M}$ [^3H]2-m-AzTax in the absence or presence of a 4-fold molar excess of unlabeled 2-m-AzTax, Taxol, Taxotere, SB-T-1214, and SB-CST-10202, followed by UV irradiation, SDS-PAGE, and fluorography. (B) Each band in (A) was image quantified by ImageJ and plotted. Results from two experiments are presented as mean $\pm$ SE. C = control.

Figure 3.

SB-T-1214 and SB-CST-10202 inhibit 2-m-AzTax photolabeling of P-gp and cause an increase in [3 H]vinblastine ([3 H]VBL) accumulation in multidrug-resistant (MDR) cells. (A) Plasma membranes prepared from drug-sensitive SKOV3 and its MDR cell line SKVLB1. (B) Plasma membranes (100 μ g) from SKOV3 and SKVLB1 cells were incubated with 1 μ M [3 H]2-m-AzTax in the presence of 40 μ M taxanes, followed by UV irradiation, SDS-PAGE, Coomassie Blue staining, and fluorography. (C) SKOV3 and SKVLB1 cells were incubated with 1 μ M [3 H]vinblastine in the presence of 40 μ M taxanes for 1 h, and steady-state [3 H]vinblastine accumulation was determined as described in the Experimental section. (D) Log(% photolabeling) plotted against log([3 H]VBL accumulation) and the correlation coefficient derived. Data for % photolabeling and for [3 H]VBL accumulation are from B and C, respectively.

Figure 4.

Increased expression of *MDR1* and/or β III-tubulin was seen in drug-resistant ovarian cancer Hey cells. SB-T-1214 is potent against Taxol- and ixabepilone-resistant Hey cells. (A) *MDR1* levels in drug-resistant Hey cells were determined by qPCR. (B) Cell lysates (20 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau S to ensure equal loading. β III-Tubulin levels in the resistant cells were determined by Western blot analysis using anti- β III-tubulin antibody (tuj-1, Covance). (C) A total of 1000–2000 cells were plated in each well of a 96-well plate. Increasing concentrations of the drugs were added 18 h after plating. IC₅₀ values were determined after 96–120 h of incubation at 37 °C, depending on the growth rate of each cell line (see Table 1). Fold resistance values for each drug were plotted for each cell line.

Figure 5.

SB-T-1214 is very potent against the MDR cell line SKVLB1. (A) SKVLB1 expresses high levels of P-gp as seen in the Coomassie Blue stained gel. (B) Cell lysates (20 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau S to ensure equal loading. SKVLB1 expresses a reduced level of β III-tubulin. IC_{50} values and fold resistance were determined (see Table 2). (C) Fold resistance values for each drug were plotted for SKVLB1 cells.

Figure 6.

Verapamil (VPL) increases sensitivity of the MDR cell line SKVLB1 to SB-T-1214. (A) Cytotoxicity of SB-T-1214 in SKOV3 and SKVLB1 cells grown in the presence of 2 μ M VPL. (B) SKOV3 and SKVLB1 cells were grown in the presence or absence of 2 μ M VPL, and IC₅₀ values for SB-T-1214 determined (see Table 3). (C) Effects of 2 μ M VPL on resistance to SB-T-1214 in SKVLB1 cells.

Table 1.

Cytotoxicity of the Next-Generation Taxanes against Drug-Resistant Ovarian Cancer Cells

	$IC_{50}^a \pm SE$ (nM)			
	Hey	Hey.Tx100	Hey.Ixab80	Hey.EpoB8
Taxol	2.7 ± 0.1 (1)	240.0 ± 6.9 (89 $\pm$ 3)	152.3 ± 13.8 (56 $\pm$ 5)	22.2 ± 0.5 (8 $\pm$ 0.2)
Taxotere	0.86 ± 0.06 (1)	41.4 ± 3.7 (48 $\pm$ 4)	16.2 ± 1.8 (19 $\pm$ 2)	8.4 ± 0.2 (10 $\pm$ 0.2)
SB-T-1214	0.58 ± 0.08 (1)	2.3 ± 0.4 (4 $\pm$ 0.6)	1.6 ± 0.1 (3 $\pm$ 0.2)	8.0 ± 0.1 (14 $\pm$ 0.2)
SB-CST-10202	0.82 ± 0.07 (1)	28.8 ± 1.1 (35 $\pm$ 1)	13.0 ± 1.1 (16 $\pm$ 1)	13.9 ± 0.3 (17 $\pm$ 0.3)

^a IC_{50} equals the concentration (nM) of drug that inhibits 50% cell growth; 1000–2000 cells were plated, and IC_{50} values determined as described in Experimental Section. Data are expressed as mean $\pm$ SE ($n = 3$). Values in parentheses are fold resistance (IC_{50} of resistant cells/ IC_{50} of sensitive cells).

Table 2.

Cytotoxicity of the Next-Generation Taxanes against Vinblastine-Resistant Ovarian Cancer Cells

	$IC_{50}^a \pm SE$ (nM)		
	SKOV3	SKVLB1	fold resistance
Taxol	2.39 ± 0.19	12590 ± 592	5416 ± 247
Taxotere	0.75 ± 0.02	3533 ± 71	4704 ± 95
SB-T-1214	0.62 ± 0.04	75.6 ± 12.3	121 ± 20
SB-CST-10202	1.15 ± 0.15	1578 ± 94	1376 ± 82

^a IC_{50} equals the concentration (nM) of drug that inhibits 50% cell growth; 1500–2000 cells were plated, and IC_{50} values determined as described in the Experimental Section. Data are expressed as mean $\pm$ SE ($n = 3$). Fold resistance was determined as IC_{50} of resistant cells/ IC_{50} of sensitive cells.

Table 3.

Effects of Verapamil on Cytotoxicity of SB-T-1214 against Vinblastine-Resistant Ovarian Cancer Cells

	$IC_{50}^a \pm SE$ (nM)		fold resistance	ratio
	SKOV3	SKVLB1		
Taxol (– verapamil)	3.85 ± 0.16	21228 ± 665	5521	
Taxol (+ verapamil)	3.99 ± 0.29	3733 ± 426	936	5.9
SB-T-1214 (–verapamil)	1.46 ± 0.15	182.0 ± 9.8	124	
SB-T-1214 (+ verapamil)	1.44 ± 0.11	46.5 ± 8.2	32	3.9

^a IC_{50} equals the concentration (nM) of drug that inhibits 50% cell growth; 1500–2000 cells were plated, and IC_{50} values determined as described in the Experimental section. Data are expressed as mean ± SE ($n = 3$). Fold resistance was determined as IC_{50} of resistant cells/ IC_{50} of sensitive cells.