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Biochemical Pharmacology 62 (2001) 1469–1480

Biochemical Pharmacology

Accompanying protein alterations in malignant cells with a microtubule-polymerizing drug-resistance phenotype and a primary resistance mechanism

Marianne S. Poruchynsky[a,*], Paraskevi Giannakakou[a], Yvona Ward[a], J. Chloe Bulinski[b], William G. Telford[a], Robert W. Robey[a], Tito Fojo[a]

[a]*NIH, The National Cancer Institute Center for Cancer Research, Bldg. 10, Rm. 12N226, 9000 Rockville Pike, Bethesda, MD 20892-1910, USA*
[b]*Columbia University, College of Physicians and Surgeons, New York, NY, USA*

Received 8 May 2000; accepted 8 May 2001

Abstract

Microtubules (MTs) are cytoskeletal components whose structural integrity is mandatory for the execution of many basic cell functions. Utilizing parental and drug-resistant ovarian carcinoma cell lines that have acquired point mutations in β-tubulin and p53, we studied the level of expression and modification of proteins involved in apoptosis and MT integrity. Extending previous results, we demonstrated phosphorylation of pro-survival Bcl-x_L in an epothilone-A resistant cell line, correlating it with drug sensitivity to tubulin-active compounds. Furthermore, Mcl-1 protein turned over more rapidly following exposure to tubulin-modifying agents, the stability of Mcl-1 protein paralleling the drug sensitivity profile of the paclitaxel or epothilone-A resistant cell lines. The observed decreases in Mcl-1 were not a consequence of G_2M arrest, as determined by flow cytometry analysis, which showed prominent levels of Mcl-1 in the absence of any drug treatment in populations enriched in mitotic cells. We also observed that a paclitaxel-resistant cell line expressed Bax at a much lower level than the sensitive parental line [A2780(1A9)], consistent with its mutant p53 status. MT-associated protein-4 (MAP4), whose phosphorylation during specific phases of the cell cycle reduces its MT-polymerizing and -stabilizing capabilities, was phosphorylated in response to drug challenge without a change in expression. Phosphorylation of MAP4 correlated with sensitivity to tubulin-binding drugs and with a dissociation from MTs. We propose that the tubulin mutations, which result in a compromised paclitaxel:tubulin or epothilone:tubulin interaction and paclitaxel or epothilone resistance, indirectly inhibit downstream events that lead to cell death, and this, in turn, may contribute to the drug-resistance phenotype.

Keywords: Drug-resistance; Mutation; Microtubules; Mcl-1; MAP4; Cell death

1. Introduction

The regulatory mechanisms that control normal cell proliferation and cell death remain balanced through orchestrated cascades of multiple interacting signaling pathways. Members of such networks exercise triggers and constraints, resulting in normal cell metabolism, motility, division, growth arrest, and proliferation. When this rhythmic scheme is perturbed, either permanently as might be envisioned in a transformed cell [1–3], or transiently as after drug challenge, the effects could provide survival advantages to the cell.

One of several obstacles to the effective treatment of cancer has been the resistance of cells to chemotherapeutic agents [2]. The ability to circumvent such barriers would be improved by the identification of the factors that contribute to the ability of the cell to survive and evade the toxic effects of a drug. With regard to apoptosis, it is well appreciated that the Bcl-2 family of proteins that mediate this process is expanding in number, and encompasses proteins facilitating cell death (e.g. Bax and Bad) and those promoting survival (e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and viral protein E1B-19K) [4,5]. When the pendulum swings towards cell death, events culminate in activation of caspases

* Corresponding author. Tel.: +1-301-496-6611; fax: +1-301-402-1608.

E-mail address: mporuch@helix.nih.gov (M.S. Poruchynsky).

Abbreviations: MTs, microtubules; MAPs, microtubule-associated proteins; MAP4, microtubule-associated protein-4; PTX, paclitaxel; EPOA, epothilone A; EPOB, epothilone B; EPOA-R, epothilone A-resistant; COL, colchicine; VCR, vincristine; and VBL, vinblastine.

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PII: S0006-2952(01)00804-8

[5], leading ultimately to cell demise [5,6]. We, as well as others, have shown that several of the pro-survival Bcl-2 family members, such as Bcl-2 and Bcl-x_L, become phosphorylated following treatment of cells with tubulin-targeting drugs [7–9]. This modification requires an uncompromised drug:tubulin interaction [7], is accompanied by cell cycle arrest, and is often followed by apoptosis. Since several family members interact to form dimers and heterodimers, the levels of each protein and their regulation have been acknowledged as bridling the "cell-death rheostat" [4,5,10]. Therefore, we studied the responses of sensitive and resistant ovarian carcinoma cells to tubulin-targeting drugs, and examined the expression of two additional members of the Bcl-2 family with opposing functions, Bax and Mcl-1, while considering the drug-resistance profile of each cell. The pro-survival family member *MCL-1* gene [4] encodes a 46-kDa protein that has sequence similarity to Bcl-2 at its carboxyl terminus [11], an overlapping intracellular localization pattern [12], and is well studied in ovarian cell differentiation and follicle atresia [11]. To assess the possible contribution of Mcl-1 protein to support a previously determined drug-resistance phenotype and promote survival, we determined its basal level of expression in each ovarian cell line and compared it with that observed after drug treatment.

Presently, an intense interest exists in the cytoskeleton as a chemotherapeutic target, since it is well recognized that its components provide a framework where signal transduction pathways can interact [13–15] and form crucial structural scaffolds that dismantle and then reform during mitosis and cytokinesis [16,17]. The latter events require the functional integrity of the MT network and the coordinated reorganization of MTs into spindles to achieve accurate chromosome segregation. Many compounds have been identified that interfere with MTs and spindle formation or maintenance, either by depolymerizing MTs (e.g. VCR, COL, nocodazole) or by polymerizing tubulin (e.g. PTX, docetaxel, epothilones, discodermolide, sarcodyctin, eleutherobin) [18]. The fidelity of MT dynamics and function throughout the cell cycle is influenced by many components, one being the action of MAPs like tau and MAP4 [19–21]. MAPs promote MT integrity, and the phosphorylation of MAPs results in their inability to stabilize MTs, altering their dynamics [19,21–26]. Given the close physical and functional relationship between MTs and MAP4, and the ubiquitous nature of the latter, we studied the level of MAP4 protein expression and its modification after treating cells with tubulin-binding drugs.

In the present study, we examined representative opposing apoptotic regulatory proteins to determine if their modification and expression might contribute to the drug-resistance phenotype. Our observations made on pro-survival proteins Mcl-1 and Bcl-x_L, pro-death Bax, and the regulation and modification of MAP4 parallel the drug sensitivity profile for each cell line. Our results suggest that although the mutations in tubulin confer drug resistance due to impaired drug:tubulin interactions, proteins mediating the apoptotic pathway or MT structure are affected indirectly, and these secondary effects might support the drug-resistance phenotype.

2. Materials and methods

2.1. Cells and culture

A2780(1A9) is a single cell clone of the human ovarian carcinoma cell line A2780. Two PTX-resistant sublines, PTX10 and PTX22, have been described previously and are maintained in 15 ng/mL of PTX and 5 μg/mL of verapamil [3]. An epothilone-A resistant cell line, EPOA-R, was selected in EPOA and maintained in 30 nM EPOA as described [27,28]. Cells were removed from the drug 5–7 days prior to an experiment. SKOV3 is a human ovarian carcinoma line. All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

2.2. Immunoblotting

Cells were lysed at 4^o in TNESV buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 25 mM sodium fluoride, and 1% NP-40). Lysates were incubated at 4^o for 15 min, centrifuged at 4^o for 15 min at 15,800 *g*, and protein concentrations determined using the Bio-Rad protein assay dye reagent. Proteins were separated by 7.5% (Mcl-1, cyclin B1, MAP4, and p53) or 16% (Bcl-x_L, Bax, and Mcl-1) SDS–PAGE, transferred to membranes, and probed with antibodies. Mcl-1 rabbit polyclonal antibody was used at 1:1000 (Santa Cruz), cyclin B1 mouse monoclonal antibody at 1:100 (Santa Cruz), and Bcl-x_L 13.6 rabbit polyclonal antiserum at 1:2500 (derived from a recent bleed following restimulation of the animal that generated previously described 13.4 antibody; provided by C. Rudin and C. Thompson, University of Chicago). Both mouse monoclonal anti-human Bax (Pan Vera Corp.) and p53 (Ab-2, Calbiochem) antibodies were used at 1:1000. MAP4 rabbit antiserum, LHB5, was provided by C. Bulinski, Columbia University, and used at a dilution of 1:1000. In some cases, blots were stripped with 0.2 M NaOH and reprobed with a rabbit anti-actin antibody (Sigma Chemical Co.) used at a dilution of 1:400. Blocking steps, antibody incubations, and blot washes (the latter including 0.05% Tween-20) were carried out in either TTBS (100 mM Tris, pH 7.5, 154 mM NaCl with 5% nonfat dry milk) or TNE (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl). Experiments that evaluated protein modification or expression under different conditions were repeated three to five times.

2.3. RT–PCR detection of Mcl-1

Exponentially growing A2780(1A9) cells, incubated with either 0, 25, 50, 100, 250, 500, or 1000 ng/mL of PTX

for 20 hr, had their total RNA isolated using RNA STAT-60 (Tel-Test, Inc.), and first strand cDNA generated by standard methods, as described previously [3]. An aliquot of each sample was amplified for 30 cycles using the primer pair: Mcl-1–5′ F, 5′-ATC TCT CGG TAC CTT CGG GA; Mcl-1–3′ R, 5′-ACT GGG ATT TAC AGA ACT CAG G; and an annealing temperature of 57°. Aliquots of the reaction products were subjected to electrophoresis on a 1% agarose gel alongside a 100-bp marker ladder and a control reaction lane containing only water.

2.4. Flow cytometry for cell cycle analysis

Exponentially growing A2780(1A9) or EPOA-R cells were harvested using trypsin and labeled with Hoechst 33342 at 2 μM in Hanks' Balanced Salt Solution (HBSS) containing 2% fetal bovine serum for 45 min at 37°. Cells were then immediately analyzed and sorted into G_0G_1, S, and G_2M phase populations using a FACSVantage high-speed cell sorter equipped with a krypton-ion laser emitting at 351 nm (100 mW) for UV excitation of the Hoechst dye. Cell cycle regions for flow cytometric cell separation were set using Hoechst 33342-labeled chick erythrocyte and calf thymus nuclei as internal controls. Width versus area discrimination was incorporated into the sorting protocol to exclude doublets. Sort purity was estimated to be greater than 95% based on pre- and post-sort bead test sorts (since immediate dissociation of the Hoechst dye following sorting made direct back analysis of cell cycle subpopulations impossible). For the A2780(1A9) cells, the G_2M, G_0G_1, and S phases represented 4.2, 63.8, and 25.6% of the total population. The cells from each phase were lysed in TNESV at 4°, and the protein concentration was determined; 50 μg of each phase was separated by 7.5% SDS–PAGE and analyzed by western blot using antibodies to Mcl-1, as described above, and mouse monoclonal anti-α-tubulin antibody DM1A (Sigma) at 1:1000.

2.5. Pulse-chase radiolabeling of cells and immunoprecipitation

Exponentially growing A2780(1A9) cells were either treated or not treated in complete RPMI medium with 250 ng/mL of PTX for a total of 18 hr. Sixteen hours into the incubation, the cells were rinsed with methionine- and cysteine-free RPMI, and then labeled for 2 hr with 245 mCi/mL of TRAN [35]S-LABEL (specific activity, 1175 Ci/mmol; ICN Biomedicals, Inc.) in the same medium plus 10% fetal bovine serum, in the presence or absence of 250 ng/mL of PTX. At the end of the labeling period, the cells were rinsed briefly and then incubated in complete medium for 0, 5, 15, 30, or 60 min in the presence of drug. Following the chase period, the medium was removed, and cells were placed on ice and lysed in 0.5 M NaCl, 0.05 M Tris, pH 7.8, containing 1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. The lysates were pre-cleared with Protein-A-Sepharose CL4B (Pharmacia) and immunoprecipitated with the anti-Mcl-1 antibody described above, except for the no-antibody control, and then Protein-A-Sepharose CL4B. Immunoprecipitated products were washed in the lysis buffer and solubilized for SDS–PAGE.

2.6. Phosphatase treatment

Exponentially growing sub-confluent cells were treated for 20–24 hr with either PTX or VCR, 150 ng/mL, before lysis at 4° in a buffer consisting of 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, with 2 mM phenylmethylsulfonyl fluoride and 100 units/mL of aprotinin. Lysates were incubated at 4° for 1 hr, and centrifuged as described previously; the protein concentrations were determined for each of the supernatants. Aliquots containing 100 μg of protein were adjusted with the 10× phosphatase reaction buffer (New England Biolabs) and 2 mM $MnCl_2$. One hundred units of λ-protein phosphatase was added, and samples were incubated at 30° for 3 hr, in either the absence or presence of phosphatase inhibitors (25 mM NaF and 1 mM Na_3VO_4). Other control samples lacking enzyme were incubated at either 30° or 4° for the same length of time prior to analysis by gel electrophoresis.

2.7. Immunofluorescence microscopy

Cells plated on glass coverslips were incubated either without drug or with 75 ng/mL of VCR or PTX for 3 hr prior to fixation in 100% methanol at –20° for 10 min. After rinsing in PBS, the coverslips were incubated for 45 min in 20% goat serum in PBS followed by incubation in rabbit LHB antibody to MAP4 for 1 hr. After three rinses in PBS containing 5% goat serum and 0.2% BSA, the coverslips were incubated for 45 min in a secondary Texas-Red conjugated donkey anti-rabbit antibody (Vector Laboratory), rinsed three times, then incubated with the mouse monoclonal anti-α-tubulin antibody DM1A (Sigma) for 45 min, rinsed again three times, and finally incubated with the secondary fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse antibody (Molecular Probes, Inc.) for 45 min. All incubations were carried out at 22°. The coverslips were rinsed in PBS and then one time briefly in distilled water prior to being mounted on glass slides using Gel/Mount (Biomeda Corp.). Representative cell fields were examined on a Zeiss Axioplan microscope using the Zeiss Plan-Apo 100×/1.4 oil immersion objective, and confocal fluorescent sections were obtained on a Zeiss Laser Scanning Microscope (LSM 510). Subsequently, three-dimensional maximal projection images were generated showing MAP4 (red), α-tubulin (green), or the merged images.

Table 1
Mutation analysis of A2780 drug-resistant clones

Cell line	β-Tubulin codon	Codon change	Amino acid substitution	p53 codon	Codon change	Amino acid substitution
A2780(1A9)		Wild-type	Wild-type		Wild-type	Wild-type
PTX10	270	TTT-GTT	Phe-Val	236(±)[a]	TAC-TGC	Tyr-Cys
PTX22	364	GCA-ACA	Ala-Thr	239	AAC-GAC	Asn-Asp
EPOA-R	274	ACC-ATC	Thr-Ileu	245(±)	GGC-GAC	Gly-Asp

[a] (±): indicates the presence of both a wild-type allele and one containing the designated mutation in p53, consistent with an apparent dominant-negative p53 phenotype [28].

3. Results

3.1. Effect of tubulin-targeted drugs on phosphorylation of Bcl-x_L and expression of Mcl-1

Cell death following exposure to an MT-active agent could occur as a result of the drug:tubulin interaction and the ensuing sequelae, and/or as a consequence of events independent of drug binding to tubulin. Development of drug resistance could then involve one or several mechanisms. The present studies were designed to examine additional changes that occur as a consequence of drug:tubulin interaction, and how these might contribute to, or be affected during, the development of drug resistance. Characteristics of the cell lines used in this study are summarized in Tables 1 and 2. In brief, we used two PTX-selected and one EPOA-selected human ovarian carcinoma cell lines, the trio possessing distinct acquired β-tubulin and p53 mutations [3,27,28] (Table 1). The cells lack any MDR1 mRNA [3,27] and have impaired PTX- or EPOA-driven tubulin polymerization both in intact cells and *in vitro* [3,27,28]. A mutant p53 codon homozygous substitution was detected for PTX22, whereas for PTX10 and EPOA-R both a wild-type allele and a mutant p53 codon were found [(+/−) Table 1] [28]. For PTX10, little wild-type p53 function was detected, which is consistent with a dominant-negative phenotype [28]. However, in EPOA-R cells, significant wild-type p53 function may be present as evidenced by a low basal level of p53, its induction by Adriamycin, and a high level of p53-dependent basal transcription [29]. The data suggest that the resistance exhibited by the three cell lines is due primarily to the compromised interaction of drug with its mutated β-tubulin target, the latter comprising 90% of the β-tubulin mRNA in A2780(1A9) cells [3]. The sensitivity profile of these cells to tubulin active agents is shown in Table 2. The PTX and EPO-A resistant cells were between 24- and 40-fold resistant to the selecting drug compared with the parental line, and their tubulin was able to polymerize, in that when another polymerizing drug was used (e.g. EPO-B), the impaired polymerization in cells or of isolated tubulin was overcome [3,28].

We observed previously that treatment of PTX-sensitive, but not PTX-resistant cells, with PTX resulted in the phosphorylation of Bcl-x_L [7]. Extending these earlier observations, we found that Bcl-x_L was also phosphorylated in A2780(1A9) sensitive cells in response to tubulin-polymerizing agents other than PTX, including EPOA and EPOB (Fig. 1A, arrowhead indicates phosphorylated Bcl-x_L). In addition, we observed that Bcl-x_L was not phosphorylated in an EPOA-R cell line after exposure to EPOA but was modified following VCR or VBL, two tubulin-active agents to which the cells retained sensitivity (Fig. 1A). The lack of Bcl-x_L phosphorylation in cells resistant to a particular drug could support the drug-resistance phenotype, since Bcl-x_L is viewed as functioning in a pro-survival capacity when unphosphorylated [9].

We also observed that the level of the pro-survival Bcl-2 family member Mcl-1 [4,5] was decreased in A2780(1A9) cells after treatment with PTX, VBL, VCR, or COL (Fig. 1B). Concomitant with mitotic arrest caused by these drugs, cyclin B1, a marker of the mitotic phase, was increased in sensitive parental cells after treatment with any of the four compounds (Fig. 1B). Using the three drug-resistant cell lines (Table 1), we demonstrated a decrease in the level of

Table 2
Cytotoxicity/resistance profile of A2780-derived clones

Cell line	PTX IC_{50} (nM)	RR[a] (PTX)	EpoA IC_{50} (nM)	RR (EpoA)	EpoB IC_{50} (nM)	RR (EpoB)	VBL IC_{50} (nM)	RR (VBL)
A2780(1A9)	2		3.5		0.25		0.25	
PTX10	47	24	1.0	0.3	0.70	2.8	0.50	2
PTX22	48	24	3.5	1.0	0.35	1.4	0.65	2.6
EPOA-R	19	10	140.0	40.0	8.0	32.0	0.75	3

[a] RR: for each drug, the relative resistance was calculated by dividing the IC_{50} for each cell line by the IC_{50} of the A2780(1A9) cells.

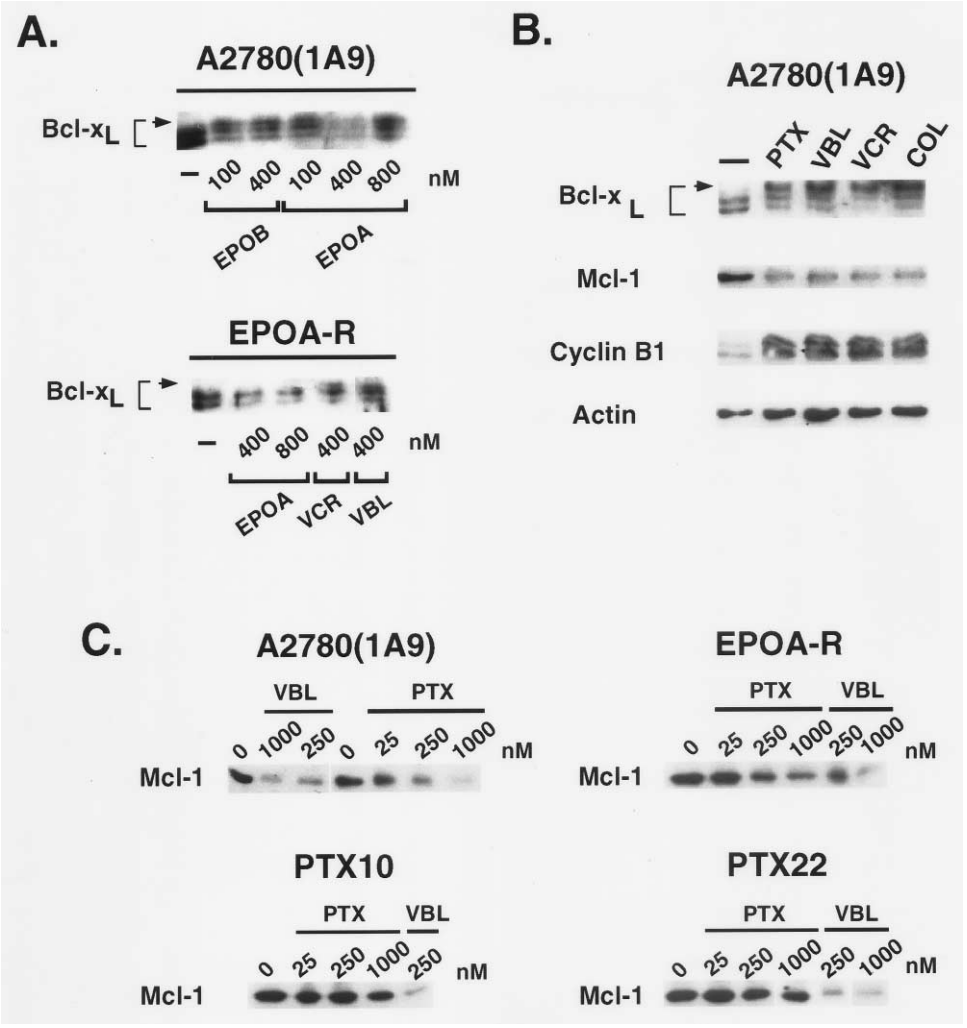

Fig. 1. Western blots displaying the effects of tubulin-targeted drugs on the phosphorylation of Bcl-x_L and the expression of Mcl-1. (A) The more slowly migrating phosphorylated Bcl-x_L form was apparent in A2780(1A9) parental and EPOA-R cells in response to drugs for which the cells are sensitive. EPOA-R cells failed to show a Bcl-x_L modification in response to EPOA, but did with VCR and VBL. The bracket indicates the immunoreactive forms of Bcl-x_L and the arrowhead points to phosphorylated Bcl-x_L (A and B). (B) A2780(1A9) cells were treated with 150 ng/mL each of PTX, VBL, VCR, or COL. After probing with anti Mcl-1, the blot was reprobed with antibodies to actin, cyclin B1, and Bcl-x_L. (C) Sensitive parental A2780(1A9), PTX-resistant (PTX10 or PTX22), and EPOA-resistant (EPOA-R) cells were treated with nanomolar amounts of the drugs indicated for 18 hr. Equal amounts of total protein were loaded for each sample, and the blot was probed with anti-Mcl-1.

Mcl-1 only after treatment with tubulin-targeted drugs for which a particular cell was sensitive (Fig. 1, B and C). A reduction in the Mcl-1 level did not occur when cells exhibiting resistance to PTX (PTX10 and PTX22) were treated with increasing concentrations of PTX (Fig. 1C), and was blunted in the EPOA-R cell line in response to PTX (Fig. 1C), consistent with the 10-fold cross-resistance of this cell line to PTX (Table 2). These observations reinforce the conclusion that an uncompromised drug:tubulin interaction is a requirement for phosphorylation of Bcl-x_L [7], and for a decrease of Mcl-1 levels. The observation that these changes do not occur in the drug-resistant cells suggests that they may contribute to cell survival.

To address the possibility that Mcl-1 protein expression is lower in mitotic cells independent of tubulin-targeted drug effects, two experiments were performed. In one study, cells arrested in G_1 and G_2 with Adriamycin were treated with PTX. A2780(1A9) parental cells were treated initially either without drug, with Adriamycin (A) (400 ng/mL), or with PTX (P) (200 ng/mL) for 12 hr, after which time fresh Adriamycin, PTX, or Adriamycin plus PTX were added for an additional 11 hr (Fig. 2A). The level of Mcl-1 was reduced only when cells were treated with PTX in the absence of Adriamycin (Fig. 2A). When cells were blocked with Adriamycin and then treated with both Adriamycin and PTX, the level of Mcl-1 protein did not decrease (Fig. 2A). Thus, in order for the level of Mcl-1 protein to decrease in response to a tubulin-targeting agent, the cell must be able to reach, and possibly be blocked, in the G_2M phase.

Flow cytometry separation of exponentially growing, untreated cells was also performed to retrieve a population enriched in mitotic cells for comparison with those in G_0G_1

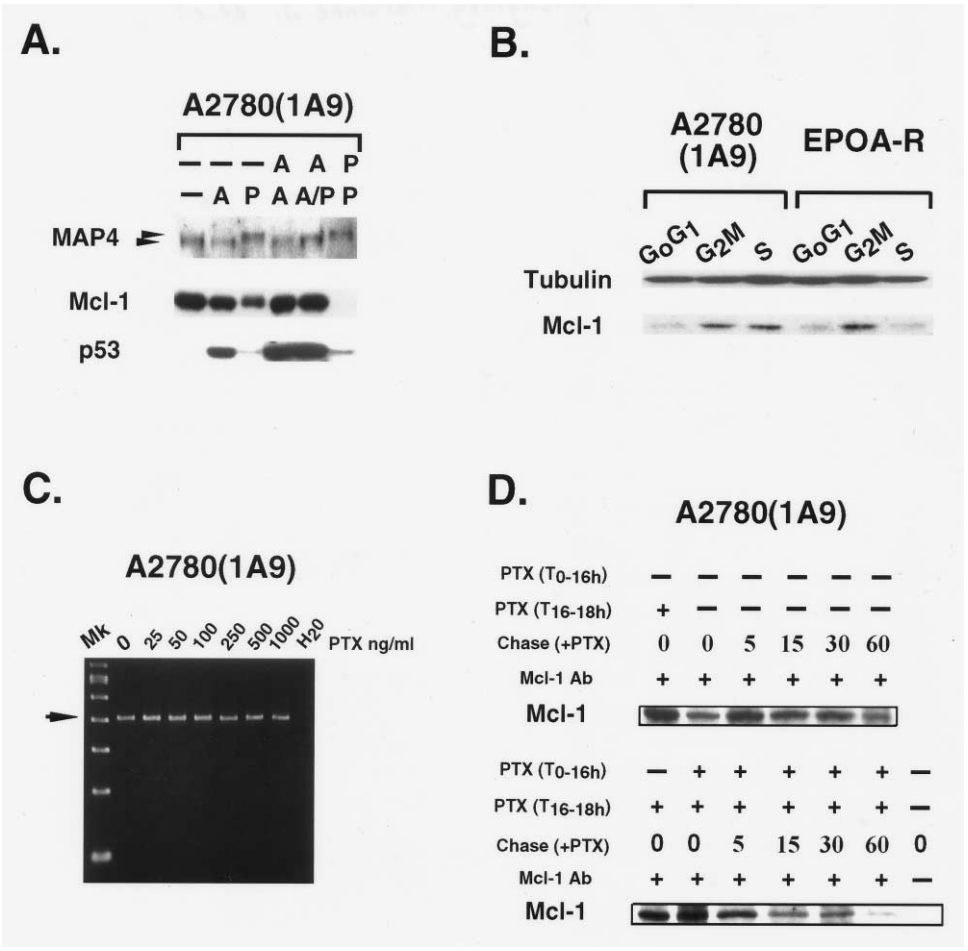

Fig. 2. Mcl-1 protein expression and RNA analysis. (A) In a two-step drug challenge, A2780(1A9) cells were incubated initially either without drug (–), with 400 ng/mL of Adriamycin (A), or with 200 ng/mL of PTX (P) for 12 hr, followed by a change of medium containing the A/P together or either drug alone for an additional 11 hr. The western blot was probed for MAP4, Mcl-1, and p53 as indicated. Upper and lower arrowheads indicate the modified and unmodified forms of MAP4, respectively. Cells treated with Adriamycin did not show a decrease in Mcl-1 protein expression. (B) Mcl-1 protein expression analysis in the absence of drug treatment, in exponentially growing sensitive parental A2780(1A9) or EPOA-resistant (EPOA-R) cells stained with Hoechst dye 33342 and separated by flow cytometry into G_oG_1, G_2M, and S populations, as described in "Materials and methods." Fifty micrograms of total protein from each cell line lysate and cell cycle phase was separated by SDS–PAGE, transferred to Immobilon, and probed with antibodies to Mcl-1 and α-tubulin. (C) RT–PCR of total RNA isolated from cells treated with increasing concentrations of PTX for 20 hr. Amplification using Mcl-1 primers was performed for 30 cycles, and the arrowhead indicates the expected (~420 bp) size of the Mcl-1 product. (D) Cells either treated (+) or not treated (–) with 250 ng/mL of PTX for 18 hr were labeled for the last 2 hr with 245 mCi/mL of TRAN [35]S-LABEL, and then chased with complete medium for 0–60 min in the presence of 250 ng/mL of PTX. Mcl-1 was immunoprecipitated from each lysate, and the radiolabeled product was visualized after SDS–PAGE.

and S phases. Flasks containing either A2780(1A9) or EPOA-R cells were harvested using trypsin, labeled with Hoechst 33342, and separated to >95% enrichment for each phase of the cell cycle, as described in "Materials and methods." For the A2780(1A9) cells, the G_2M, G_0G_1, and S phases represented 4.2, 63.8, and 25.6% of the total population, respectively, with similar percentages for EPOA-R cells (data not shown). Mcl-1 and tubulin protein levels were analyzed by western blots for cell lysates in each phase (Fig. 2B). For both cell lines, the populations enriched in cells in the G_2M phase had levels of Mcl-1 protein that were greater than or equal to that of the cells in the other phases of the cell cycle, while the α-tubulin level remained fairly constant (Fig. 2B). Thus, in spite of the enrichment in cells with G_2M DNA content, no decrease in Mcl-1 expression was observed in the absence of drug treatment; indeed, if anything, an increase in its expression was noted in the EPOA-R cells (Fig. 2B). The results from these two experiments suggest that Mcl-1 protein expression decreases during the G_2M phase of the cell cycle only in the presence of tubulin-targeting drugs. The lower level of Mcl-1 protein was not a consequence of the down-regulation of Mcl-1 RNA. RT–PCR of RNA prepared from exponentially growing A2780(1A9) cells that were incubated for 20 hr with 0, 25, 50, 100, 250, 500, or 1000 ng/mL of PTX displayed almost equivalent Mcl-1 product levels for each sample (Fig. 2C) and failed to parallel the marked decrease observed for Mcl-1 protein.

By contrast, Mcl-1 protein turned over more quickly in the presence of PTX in sensitive cells than in the absence of

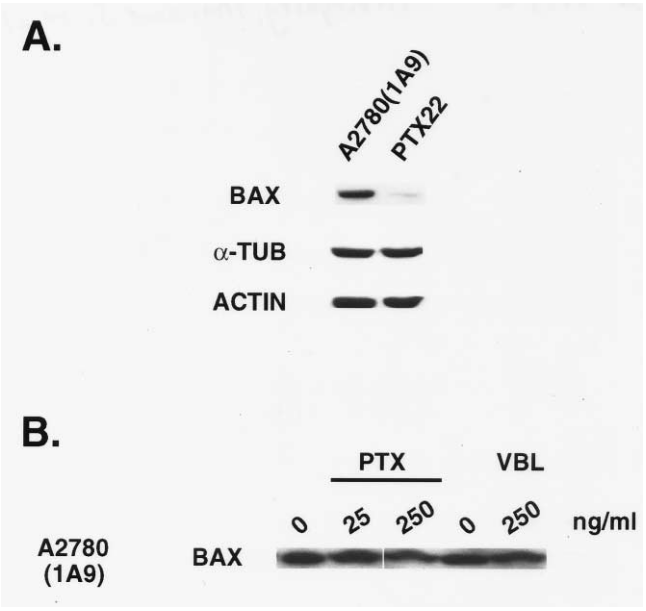

Fig. 3. Bax protein expression in response to treatment with tubulin-targeting drugs. (A) Western blot of lysates from untreated parental A2780(1A9) [wild-type p53] and PTX-resistant PTX22 (mutant p53) cell lines, first probed with anti-Bax antibody and then reprobed with anti-α-tubulin (α-TUB) and anti-actin. Equal amounts of total protein were loaded in each lane. (B) Unchanging Bax protein level in lysates from A2780(1A9) cells treated with either PTX or VBL, ranging in concentration from 25 to 250 ng/mL, for 24 hr. Equal amounts of total protein were loaded in each lane, and the blot was probed with anti-Bax.

drug (Fig. 2D). The half-life of Mcl-1 protein was approximately 15 min in cells treated with 250 ng/mL of PTX for 18 hr, compared with a half-life of at least 1 hr in untreated cells (Fig. 2D). This was determined when cells either treated or not treated with PTX were labeled with TRANS [35]S-LABEL for the last 2 hr of the 18-hr drug-incubation period, and chased for 0, 5, 15, 30, or 60 min in the presence of drug (Fig. 2D). These results indicate that the pro-survival Mcl-1 protein is degraded more quickly in sensitive cells following treatment with a tubulin-targeting drug.

3.2. Endogenous Bax protein expression in a PTX-resistant ovarian carcinoma subline

We also conducted a limited study of a pro-apoptotic protein, Bax, a representative member of this group [5] whose expression can be induced by wild-type p53 [1,30] and which forms heterodimers with pro-survival Bcl-2 family members. When quantitated by densitometry of western blots, the PTX-resistant PTX22 cell line displayed a lower endogenous level of Bax expression [26% in comparison with the parental line A2780(1A9) arbitrarily assigned a value of 100% (Fig. 3A)], corresponding to the mutant p53 status of PTX22 (Table 1). Also, the PTX10 cell line displayed an intermediate Bax level, 50% (data not shown). That this Bax adaptation in the drug-resistant cells was a

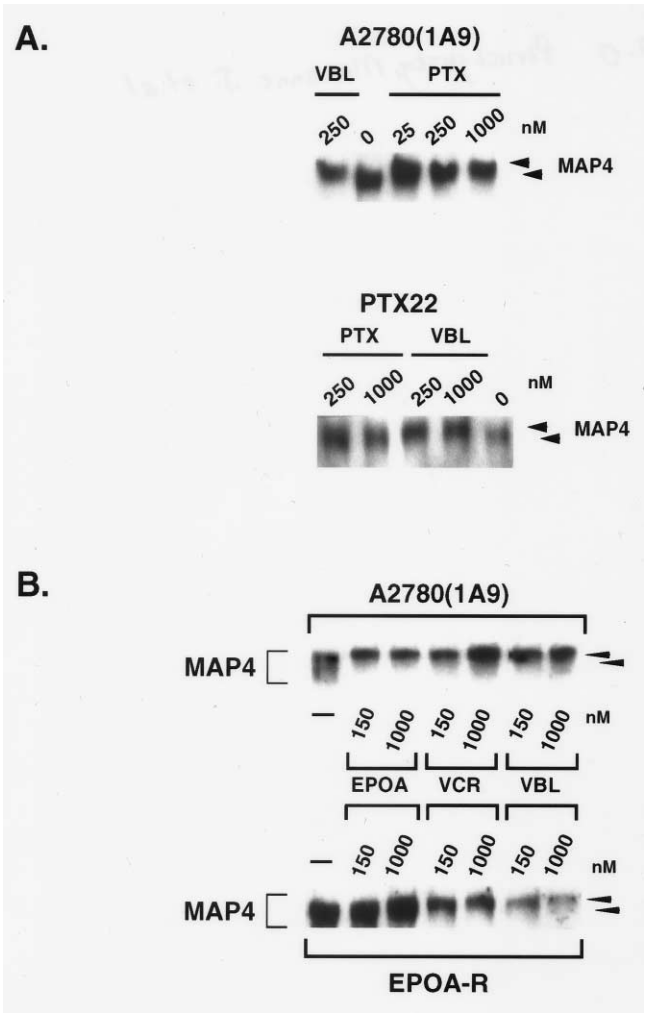

Fig. 4. Post-translational modification of MAP4 after treatment with tubulin-active agents. In panels A or B, sensitive parental cells [A2780(1A9)] or resistant [PTX22 or EPOA-R] cells were treated with either PTX, VBL, EPOA, or VCR, at the indicated concentrations for 20 hr. Upper and lower arrowheads indicate the modified and unmodified forms, respectively, of MAP4, the former occurring only when cells were sensitive to drug.

fixed change, and not an acute response to drug exposure, was confirmed by showing unchanged Bax protein expression levels in the A2780(1A9) cell line after a 20- to 24-hr incubation with either PTX or VBL, in the concentration range of 25 to 250 ng/mL (Fig. 3B). Bax also remained at the baseline levels in PTX22 and PTX10 cells when treated for 20–24 hr with up to 1000 ng/mL of either drug (data not shown).

3.3. Post-translational modification of MAP4 after treatment with PTX or VBL

MT dynamics are modulated by MAPs [16,19,21,22], some of which are phosphorylated during specific portions of the cell cycle [23–26,31–33]. Phosphorylation of MAP4 during normal mitosis is accompanied by MT reorganization [16,21–24,26], and several studies have noted a de-

creased affinity of MAP4 for MTs under these circumstances [19,23,26]. When we examined MAP4 in parental A2780(1A9) cells treated for either 12 or 23 hr with Adriamycin, a drug known to arrest cells in G_1 and G_2, we observed no alteration in MAP4 mobility (Fig. 2A). However, an increase in the size of MAP4 was observed following PTX treatment for either 12 or 23 hr, indicating that MAP4 was likely modified during the M phase under these conditions (Fig. 2A). No substantial change in the level of MAP4 expression was observed after treatment of this cell line with either Adriamycin or PTX (Fig. 2A). Vigorous induction of wild-type p53 following Adriamycin treatment and a modest response after PTX treatment (Fig. 2A) are consistent with the response of wild-type p53 to these agents [1,30].

Modification of MAP4 to a more slowly migrating form was discernible in A2780(1A9) cells after treatment for 20 hr with VBL, PTX, EPOA, or VCR (Fig. 4, A and B, upper arrowheads). In the three drug-resistant cell lines with acquired tubulin mutations (PTX22, Fig. 4A; EPOA-R, Fig. 4B; and PTX10, data not shown), MAP4 was modified only after treatment with drugs to which the cells were sensitive, such as VCR or VBL, or very high concentrations of PTX or EPOA. Since the observed protein alterations correlated well with cellular sensitivity to drug and the drugs utilized arrest cells in mitosis, it is likely that processing of MAP4 occurs then.

MAP4 has multiple potential sites for phosphorylation, and its electrophoretic mobility is altered following this post-translational modification [21,23,26]. To confirm that the mobility shift observed for MAP4 following drug treatment with tubulin-active drugs was due to phosphorylation, cell lysates derived from cells treated with either PTX or VCR were incubated with λ-protein phosphatase, which has specificity for cleavage of phosphate groups appended to the amino acids serine, threonine, or tyrosine. The enzyme-treated samples were compared to lysates not treated with enzyme and to lysates treated in the presence of phosphatase inhibitors. When phosphatase was added to the lysates of drug-treated A2780(1A9) cells in the absence of inhibitors and then incubated at 30° for 3 hr, the most slowly migrating form of MAP4 (Fig. 5, upper arrowhead) was absent. The disappearance of this form of MAP4 was accompanied by the appearance of a more rapidly migrating form (Fig. 5), intermediate in position between the unmodified rapidly migrating form (Fig. 5, lower arrowhead) and the largest form of MAP4 (Fig. 5, upper arrowhead). Moreover, when lysates were incubated with neither enzyme nor phosphatase inhibitors at 30° (Fig. 5), or in the presence of inhibitors at either 4° (Fig. 5) or 30° (data not shown), the position of the largest size MAP4 band was unaltered, indicating that cleavage was a consequence of enzyme treatment. Thus, the modification observed for MAP4 following treatment of sensitive cells with MT-targeted drugs appears to be due to phosphorylation.

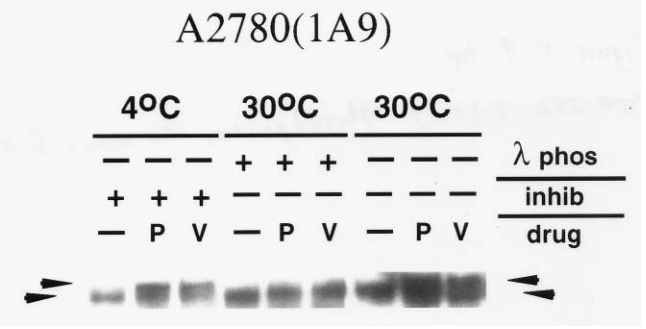

Fig. 5. λ-Protein phosphatase digestion susceptibility of the post-translational modification of MAP4 observed in cells after treatment with tubulin-targeting drugs. Untreated A2780(1A9) cells (–), or those treated with either 150 ng/mL of PTX (P) or VBL (V), were lysed in the presence (+) or absence (–) of phosphatase inhibitors and incubated at either 4° or 30° with (+) or without (–) 100 units of λ-protein phosphatase for 3 hr. Upper and lower arrowheads indicate the phosphorylated and unmodified forms of MAP4, respectively. The phosphatase-digested intermediate-sized MAP4 product migrated at a position between the two arrowheads.

3.4. Loss of MAP4 co-localization with tubulin

It is widely held that phosphorylation of MT-stabilizing MAP4 mediates its dissociation from normal MTs [16,23, 24,26,31], presumably resulting in altered MT dynamics and reorganization [16,21,23,24,31]. Since we observed MAP4 phosphorylation after treatment with either MT-depolymerizing or -polymerizing drugs, we studied whether this modification was accompanied by a dissociation of MAP4 from MTs. In untreated A2780(1A9) and PTX22 cells (Fig. 6), most MAP4 co-localized with MTs in interphase cells and on spindle MTs in normal mitotic cells [A2780(1A9), Fig. 6]. Some MAP4 was also present diffusely in all untreated cells. However, when A2780(1A9) cells were treated with 75 ng/mL of PTX for as little as 3 hr, MAP4 became diffuse and did not co-localize with α-tubulin, the latter appearing disorganized or as multiple asters in the rounded cells which predominated (Fig. 6). In contrast, when PTX22 cells were treated for 3 hr with 75 ng/mL of PTX (Fig. 6), cells retained their flat morphology, and MAP4 remained largely co-localized with MTs. When PTX22 cells were treated with 75 ng/mL of VCR for as little as 3 hr, MT depolymerization occurred, and MAP4 and α-tubulin localization became very diffuse and less coincident in both cell lines. With higher concentrations of PTX and longer incubation times, we observed MT bundling in A2780(1A9) cells with flat morphology (data not shown). The same morphological results as obtained for A2780(1A9) cells were found when we examined another sensitive human ovarian carcinoma line, SKOV3 (data not shown).

Thus, treatment with MT-active agents results in MAP4 phosphorylation and dissociation from MTs, provided the cells are drug sensitive. Where resistance exists, MAP4 remains associated with MTs. This failure to dissociate MAP4 from MTs is a marker of drug insensitivity and may

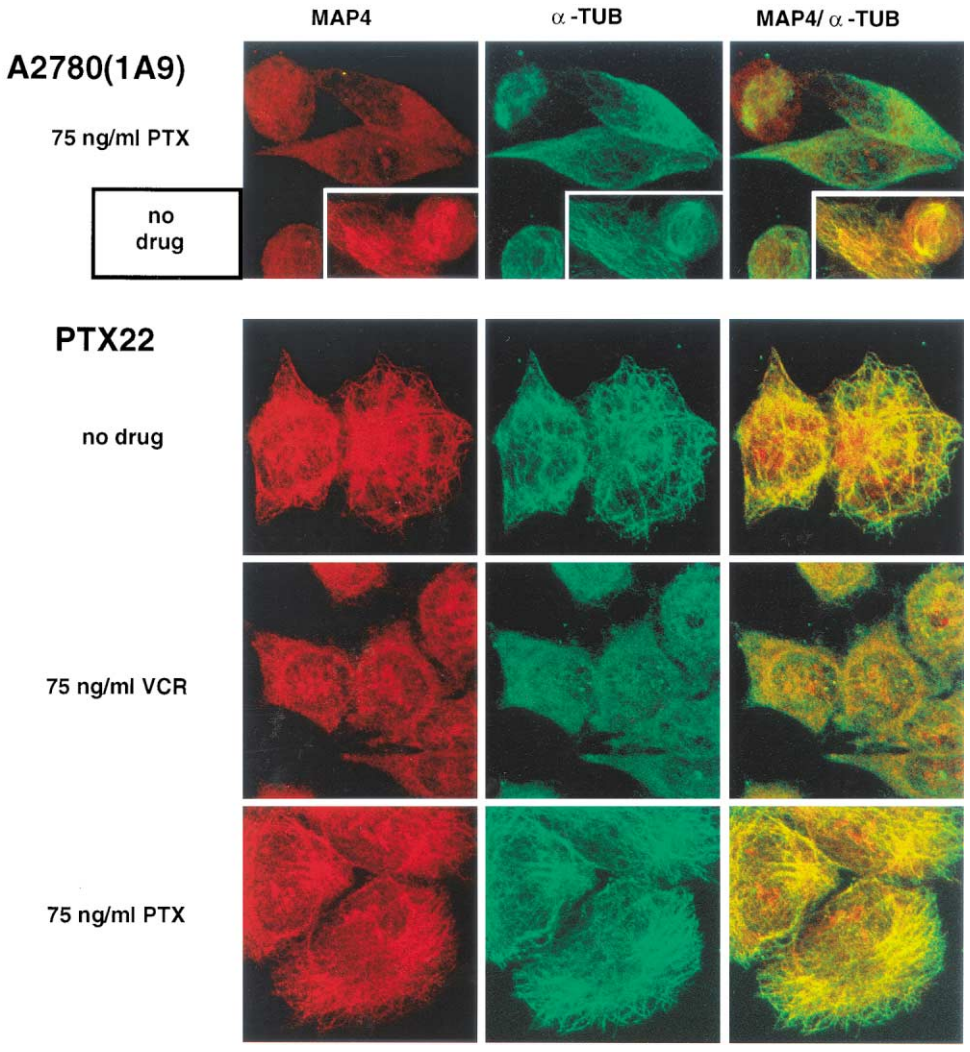

Fig. 6. Confocal double-label immunofluorescent localization of MAP4 and tubulin in treated or untreated sensitive parental or PTX-resistant cells. A2780(1A9) or PTX-resistant PTX22 cells were incubated without drug or in the presence of 75 ng/mL of VCR or PTX for 3 hr prior to fixation in methanol. Cells were then incubated sequentially in a rabbit antibody to MAP4, a secondary Texas-Red conjugated donkey anti-rabbit antibody, a mouse monoclonal anti-α-tubulin antibody, and finally, a secondary FITC conjugated sheep anti-mouse antibody. The confocal images show the localization for either the MAP4 (red) or α-tubulin (green) antibodies alongside the merged image displaying the localizations for both proteins (MAP4/α-tub) under the drug conditions indicated. For each of the cell lines in the absence of drug, there was a high degree of co-localization of the MAP4 and α-tubulin antibodies (PTX22 shown and A2780(1A9) insets). Each of the cell lines was sensitive to VCR and showed loss of microtubular structure and dispersal of the localization patterns to that of non-coincidental diffuse cytoplasmic staining (PTX22 shown). When the sensitive A2780(1A9) cells were treated with 75 ng/mL of PTX for 3 hr, the staining pattern for MAP4 became much more diffuse, whereas the localization of α-tubulin appeared as a disorganized MT network or concentrated in multiple asters in rounded cells. In contrast, resistant PTX22 cells undergoing the same PTX treatment showed extensive co-localization of MAP4 and α-tubulin, retention of tubule structures, and few mitotic cells.

also enhance the capacity of a cell to tolerate drug and retain normal cell architecture, further augmenting the drug-resistant phenotype.

4. Discussion

Chemotherapeutic strategies against many cancers utilize drugs such as the vinca alkaloids (VCR, vinorelbine), COL, epothilones, PTX, and others, which, by targeting cytoskeletal elements like tubulin, impede cell division, proliferation, signaling, motility, and transport, culminating in cell death. The ability to bypass the resistance of a malignant cell to chemotherapeutic agents would be a significant accomplishment in the clinical setting and requires elucidating the factors contributing to resistance. Although drug efflux pumps [2], or mutations that hinder drug:target interaction [3,28], have been identified, other proteins distal to the target may support the resistant phenotype. The role of the *BCL-2* gene family [4,5] in promoting or inhibiting programmed cell death has been characterized [5,34–37]. We noted in our previous studies [7,8] that two members of this family, Bcl-x_L and Bcl-2, that normally exhibit pro-viability functions were phosphorylated after treatment of sensitive

cells with a variety of MT-targeting compounds. In the present study, we demonstrated changes in several proteins that may impact cell survival, each involved in either MT integrity or programmed cell death. They highlight the consideration of events downstream of a mutation in a target that abrogates drug:tubulin interaction.

Initially cloned from differentiating ML-1 human myeloblastic leukemia cells [11], the viability-promoting effects of Mcl-1 can be more transient than those of Bcl-2 [12,38–40]. Unlike other family members (Bcl-2 or Bcl-x_L), the Mcl-1 protein sequence exhibits PEST motifs (proline, glutamate, serine, and threonine residues) that are present in other short-lived proteins and may account for its rapid turnover in some cell types [11]. Although a variety of DNA-damaging agents increase expression of Mcl-1 [39], the effects of MT-targeting drugs have not been evaluated. In the present study, we observed a decrease in the level of Mcl-1 protein after treating ovarian carcinoma cells with tubulin-targeting drugs. Our data indicate that the decrease in Mcl-1 protein was not due to the down-regulation of Mcl-1 RNA, or to decreased protein expression during mitosis, but was a result of increased turnover after a successful drug:tubulin interaction and concomitant mitotic arrest. The decrease in Mcl-1 expression, which could hasten progression towards death, was not observed in PTX-resistant cells treated with PTX (Fig. 1C), or in EPOA-R cells treated with EPOA (data not shown). This suggests that the unaltered Mcl-1 level was a consequence of the impaired drug:tubulin interaction, extending our previous observations with Bcl-2 and Bcl-x_L. A decrease of the Mcl-1 pool via any mechanism could affect the viability of a cell directly (e.g. at the mitochondrion) or indirectly via its association with Bax, mitigating the pro-death capabilities of Bax that are exercised when it is in its uncomplexed form [4,5]. Together with our observations on decreased endogenous Bax protein expression in the resistant cells, maintaining the Mcl-1 level in these cells after drug treatment could thus conceivably contribute to cell survival. The alterations observed for Mcl-1 and Bcl-x_L are secondary to drug:tubulin interaction and not a result of changes in the pathways themselves, since treatment with drugs that can interact "effectively" with tubulin results in unimpeded downstream protein modifications.

Recent studies highlight the important contribution of the Bcl-2 family members Bax and Mcl-1 in cancer cell survival in response to drugs [34–36,41] and review Bcl-2 in response to antitumor therapy [37]. For example, stable over-expression of Bax enhanced paclitaxel cytotoxicity in the SW626 human colon cancer cell line [42] and its under-expression in ~40% of human ovarian cancer tumor specimens correlated with the prediction of an inferior PTX response rate and shortened disease-free survival [35]. Wild-type p53 trans-activates the promoters of several proteins, including Bax [1,43]. Our studies showed that the endogenous level of Bax protein expression was higher in parental A2780(1A9) cells than in its PTX-resistant sublines, PTX22 and PTX10 (not shown), and correlated with p53 status [28,29].

We also examined the modification and expression of MAP4, an MT-associated protein with MT-stabilizing properties, conserved throughout evolution, and ubiquitously expressed in mammalian tissues [13,16,17,21,44], as another variable that might impact survival after treatment with tubulin-targeting compounds. Our observation that MAP4 is modified to a form susceptible to λ-protein phosphatase when cells are sensitive to tubulin-active drugs is consistent with MAP4 phosphorylation. It is likely that MAP4 phosphorylation normally occurring during mitosis was undetectable by western blotting since the percentage of mitotic cells was low (~4%) in the absence of drug. In HeLa cells, MAP4 is phosphorylated primarily during mitosis by $p34^{cdc2}$ kinase but may also be phosphorylated to some extent during interphase [32]. The phosphorylation of Bcl-x_L and MAP4 that we observed is also compatible with drug-induced mitotic arrest in the majority of cells [8], as indicated by the increase in expression of a marker of the mitotic phase, cyclin B1, when cells were treated with tubulin-targeted agents. Several studies have suggested that phosphorylation of MAP4 reduces the ability of MAP4 to stabilize MTs [23,31,32], some proposing that MAP4 dissociates from MTs [19,23], while others think it is more loosely bound [31]. Indeed, studies on the dynamics of individual MTs demonstrated a net decrease in MT stability after MAP4 phosphorylation [31]. In the present study, we observed a loss of MAP4 co-localization with MTs, accompanied by their disorganization and multiple aster formation, as assayed by double-label immunofluorescence following treatment of sensitive cells for 3 hr with 75 ng/mL of PTX. However, the co-localization of MAP4 and MTs was retained in the resistant PTX22 cells treated with PTX, and lost when either cell line was treated with VCR, the latter accompanied by dissolution of the MT network. Thus, in response to drugs, the cells are sensitive to the phosphorylation of MAP4 by a mitotic kinase, and may promote progression towards death by inhibiting normal MT/MAP4 association, functional spindle assembly, and chromosome movement. When treating cells with very low concentrations (nM) of PTX for several hours, others have observed an overall inhibition of MT dynamic instability associated with aberrant mitotic spindles and no progression to anaphase [45]. It is unknown whether identical, unique, or a subset of MAP4 sites are phosphorylated when cells normally reach M phase, compared to their undergoing treatment with tubulin-targeting drugs resulting in mitotic arrest. However, the data clearly show that MAP4 phosphorylation is a consequence of an unperturbed drug:tubulin interaction, since phosphorylation occurred in cells with mutant tubulin when effective drug:tubulin interactions were achieved. The significance of MAP4 phosphorylation is further emphasized by a study reporting that mutant nonphosphorylatable MAP4, but not wild-type MAP4, hyperstabilizes spindle MTs and inhibits chromosome movement [23]. Although

additional studies are needed to understand all the consequences of tubulin-active drugs on the interactions of MAPs with MTs, our data are in agreement with published findings indicating that unphosphorylated MAP4 makes MTs more stable and that phosphorylated MAP4, post-drug treatment in our case, appears to make MTs less stable.

In summary, our results suggest that interaction of a tubulin-active drug with its target activates numerous downstream events that might contribute to cell death. Mutations in tubulin that initially impair the drug:target interaction in PTX- or EPOA-R cells prevent these distal occurrences normally associated with cell cycle arrest and eventual death after drug treatment, and instead may facilitate cell survival.

Acknowledgments

The authors thank Zhirong Zhan for her technical assistance.

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