Proteomics of cancer cell lines resistant to microtubule stabilizing agents

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Abstract

In spite of the clinical success of microtubule interacting agents (MIAs), a significant challenge for oncologists is the inability to predict the response of individual cancer patients to these drugs. In the present study, six cell lines were compared by 2D DIGE proteomics to investigate cellular resistance to the class of MIAs known as microtubule stabilizing agents (MSAs). The human lung cancer cell line A549 was compared to two drug-resistant daughter cell lines, a Taxol resistant cell line (AT12) and an epothilone B (EpoB) resistant cell line (EpoB40). The ovarian cancer cell line Hey was compared to two drug-resistant daughter cell lines, an EpoB resistant cell line (EpoB8) and an ixabepilone resistant cell line (Ixab80). All 2D DIGE results were validated by Western blot analyses. A variety of cytoskeletal and cytoskeleton-associated proteins were differentially expressed in drug resistant cells. Differential abundance of 14-3-3σ, galectin-1 and phosphorylation of stathmin are worthy of further studies as candidate predictive biomarkers for MSAs. This is especially true for galectin-1, a β-galactose-binding lectin that mediates tumor invasion and metastasis. Galectin-1 was greatly increased in EpoB- and ixabepilone-resistant cells and its suppression caused an increase in drug sensitivity in both drug-sensitive and -resistant Hey cells. Furthermore, the growth medium from resistant Hey cells contained higher levels of galectin-1, suggesting that galectin-1 could play a role in resistance to microtubule stabilizing agents.
Introduction

The microtubule interacting agents (MIAs) are a successful class of cancer drugs [1, 2]. Several MIAs have been clinically approved and others are under development [1-3]. In spite of the success of the MIAs, a significant challenge for clinicians is their inability to predict the response of individual patients to the drugs, calling for further research into resistance to MIAs [1].

The principal mechanism of MIAs is to inhibit normal microtubule (MT) function and thereby disrupt cell division and induce cell death [1]. The targets of the MIAs are either the MTs themselves or the free heterodimers made up of α- and β- tubulins. Differential expression of tubulin isotypes, tubulin mutations and post-translational modifications (PTMs) of tubulin have been associated with drug resistance [1]. In particular, in several cancers overexpression of βIII-tubulin is associated with resistance to MIAs [1]. Certain MT-associated proteins (MAPs) have also been implicated in resistance to MIAs, including MAP2, MAP4, tau and stathmin [1], and recently, the protein family of kinesins [4]. However, molecules that are not associated with the MTs have also been associated with resistance to MIAs. Overexpression of the multidrug transporter P-glycoprotein can account for resistance to some MIAs [1, 2]. MIAs can also induce apoptosis through members of the BCL2 family [5] and recently the pro-survival protein MCL1 was shown to be a regulator of apoptosis triggered by certain MIAs [6]. The complex and multifaceted molecular mechanisms that underlie resistance to MIAs is little understood and no candidate predictive biomarker for any MIA has been advanced into prospective validation.
The aim of this study was to identify proteomic changes related to \textit{in vitro} resistance to MIAs, specifically, to three microtubule-stabilizing agents (MSAs), Taxol, epothilone B (EpoB) and ixabepilone. These drugs induce tubulin polymerization in the absence of GTP and cause microtubule stabilization and bundling [7]. Taxol is a successful cancer drug that has been approved for treatment of a variety of malignancies. Ixabepilone was recently approved for treatment of metastatic breast cancer and patupilone (epothilone B, EPO906) has been considered as a promising first-line alternative for the treatment of high-risk ovarian cancers with increased levels of βIII-tubulin and poor response to standard Taxol-cisplatin chemotherapy [8]. Interestingly, the epothilones have been shown to maintain activity against multidrug-resistant cell lines that are resistant to Taxol [9]. A biomarker that could predict resistance against Taxol or an EpoB analogue (such as Ixabepilone) would be of substantial clinical interest.

Identifying molecular aberrations related to resistance to a specific drug is challenging. A detailed comparison of many independent proteomic studies of drug resistance in cell culture revealed that the same proteins are often altered in cell lines that are resistant to different drugs [10]. These commonly observed changes may be associated primarily with an unspecific response related to cellular stress. To pinpoint proteomic changes related to resistance to a specific drug, a comparative study of six selected cell lines were carried out. Our study includes one cell line resistant to Taxol, two cell lines resistant to EpoB, and one cell line resistant to the EpoB derivative ixabepilone, as well as two drug-sensitive parental cell lines. We highlight proteomic aberrations that we believe are worthy of further investigation as candidate predictive biomarkers and as important players in MIA resistance.
Materials and Methods

Cell lines

Cells were grown in RPMI 1640 containing 10% fetal bovine serum. A549 was obtained from ATCC in 1996 and Hey cells from Dr. Gil Mor, Yale Medical School, in 2004. Low passage number cells were used for all experiments. A549 was not authenticated by small tandem repeats (STR) profiling. Resistant cell lines were isolated in authors’ laboratory. A549-T12 (AT12), A549.EpoB40 (EpoB40), Hey.EpoB8 (EpoB8) and Hey.Ixab80 (Ixab80) were maintained in 12 nM Taxol, 40 nM EpoB, 8 nM EpoB or 80 nM ixabepilone, respectively. Hey and EpoB8 cells have a 100% STR profile match.

Preparation of cell Lysates

Cells from approximately ten 100 mm culture dishes were lysed in 200 µl lysis buffer containing 30 mM Tris, pH 8.5, 7M Urea, 2M Thio-Urea, 4% CHAPS, protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor cocktail (Calbiochem). The lysed cells were sonicated on ice followed by centrifugation at 12,000xg for 30 min at 4°C.

Biochemical fractionation

The MT pellet and the tubulin-depleted fractions were prepared as described [11]. In brief, the cell pellets were resuspended in MES glutamate buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid, pH 6.8, 0.5 mM MgCl2, 1 mM EGTA, 0.1 M glutamate), including protease inhibitors and 1 mM DTT, followed by sonication and centrifugation. The 120,000xg supernatant of the cell lysate was incubated with 20 µM Taxol and 1 mM GTP at
37°C for 30 min. The solution was layered on a 20% sucrose cushion and centrifuged at 30,000xg for 30 min at 37°C. The supernatant, designated as the tubulin-depleted lysate, and the pellet containing the MTs and associated proteins were collected. The MT-associated proteins were dissociated from the pellet by high salt wash and the MTs were pelleted again as described above.

**2D DIGE**

Samples were prepared using the 2D Clean-up kit (2D Quant, GE Healthcare) and the protein concentrations were determined by absorption assay (GE). Prior to 2D DIGE analysis all samples were analyzed by SDS-PAGE to ascertain purity and estimate relative total protein abundance. A sample volume corresponding to 60 µg of total protein was treated again by the 2D Clean-up kit and the protein was resolubilized in 30 µl sample buffer (30 mM Tris, 7M Urea, 2M Thio-Urea, 4% CHAPS, pH 8.5). Samples were labeled with Cydye (GE) as described [12]. Isoelectric focusing was done with 24 cm pH 4-10 non-linear IPG strips on the Ettan IPGhorII (GE) and the second dimension separation was performed on 13% SDS-PAGE gels (JULE Biotechnology). The experiment was done in two batches of three gels; one batch for the A549 cells and one for the Hey cells (see Figure 1). Each gel included two experimental cell lines and one standard sample labeled with Cy2. Each sample was analyzed in triplicate giving a total of 18 gel images for analysis. The 2D DIGE analysis of the samples obtained from biochemical fractionation of the A549 cell series was performed as described for the total lysates and this experiment included nine samples, including three standards, and was run on three gels in one independent batch.

**MALDI TOF/TOF MS**
After scanning, the 2D DIGE gels were stained with GelCode Blue Safe Protein Stain (Thermo Scientific). Selected spots were excised, treated by in-gel trypsin digestion, the peptide extract was desalted and proteins were identified on a AB Sciex 4800 MALDI-TOF mass spectrometer (Applied Biosystems) and by the database fitting program Mascot (Matrix Science) as described [12]. Criteria for positive identification were a minimum of two significant peptides and an overall protein confidence of p<0.05. In addition, there was good agreement between the observed and the theoretical mass and pI for all identified proteins in the 2D gel.

**Western blot analysis**

Denatured cell lysates were prepared using a buffer containing 10 mM Tris-HCl, pH 7.5, and 1% SDS. Equal amounts of proteins in the lysates were resolved by SDS-PAGE and expression levels of a variety of proteins identified in the 2D-DIGE studies were determined by Western blot analyses using antibodies listed in Table S1.

**Suppression of Galectin-1 expression by RNA interference and cytotoxicity**

Cells were transfected for 24 h with 30 nM galectin-1 siRNA (Santa Cruz) and 30 nM non-targeting siRNA (Thermo Scientific) using the transfection reagent Lipofectamine RNAiMAX (Invitrogen). Transfected cells were re-plated in 96-well culture dishes at 1,000-2000 cells/well and treated with increasing concentrations of drugs. Sulforhodamine B (SRB) cytotoxicity assays were performed after 72-96h. Galectin-1 levels after siRNA transfection were determined by Western blot analysis.
Results

2D DIGE proteomics of cancer cell lines resistant to MSAs

The human lung cancer cell line A549 was compared to two drug-resistant daughter cell lines; an epothilone B resistant cell line (EpoB40) and a Taxol resistant cell line (AT12). The ovarian cancer cell line Hey was also compared to two drug-resistant daughter cell lines; an epothilone B resistant cell line (EpoB8) and an ixabepilone resistant cell line (Ixab80) (see Fig. 1 for overview of the experimental design).

EpoB40 and AT12 cell lines are approximately 100-fold and 9-fold resistant to EpoB and Taxol, respectively [13, 14]. EpoB40 harbors a Gln to Glu mutation at residue 292 that is situated near the M-loop of ß1-tubulin. AT12 has a heterozygous mutation in Kα1-tubulin at residue 379 (Ser to Ser/Arg) and is dependent on Taxol, requiring a minimum of 2 nM Taxol to maintain normal growth. Neither cell lines expressed the MDR gene product P-glycoprotein [13, 15]. EpoB8 is 20-fold resistant to EpoB [16], and no mutations were found in its α- or β-tubulin. Ixab80 is approximately 7-fold resistant to ixabepilone and harbors a ß-tubulin mutation in Helix 5 (unpublished data from our laboratory).

Triplicate samples of the proteins from the A549-series (A549, EpoB40 and AT12) and the Hey-series (Hey, EpoB8 and Ixab80) were analyzed by 2D DIGE. Overall, the 2D DIGE profiles of the A549 and Hey series were quite similar and all protein spots referred to in this study were detected in all cell extracts. Each drug resistant cell line (n=3) was compared to the drug-sensitive parental cell line (A549 or Hey) (n=3) and protein spots that were significantly different were identified (>2.0-fold difference in mean & p<0.05). We present and validate the
proteomic changes identified by 2D DIGE with particular focus on candidate predictive biomarkers (Table 1, Table S2 and Fig. 1).

**Tubulins**

To confirm the presence of the charge-altering mutation βGln292Glu that we previously identified in the EpoB40 cells [13, 17], triplicates of tubulin-depleted fractions, as described in Materials and Methods, from A549 and EpoB40 cells were compared by 2D DIGE. This procedure leaves behind a smaller pool of non-polymerized tubulin and does not polymerize specific β-tubulin isotypes [18], allowing for improved resolution of the α- and β-tubulin spots. The relative migration pattern of α- and β-tubulin by 2D electrophoresis (2DE) was in agreement with the literature (Fig. 2A and 2B): mammalian α-tubulin has a slower electrophoretic mobility than β-tubulin and the average theoretical pIs of seven α-tubulin and eight β-tubulin isotypes present in humans is 5.1 (pI: 4.9-5.7) and 4.9 (pI: 4.8-5.1), respectively [18]. In the tubulin-depleted fractions, the two left-most β-tubulin spots were significantly increased (>2.0 fold & p<0.05) in EpoB40 versus A549 (Fig. 2C), whereas the right β-tubulin spot (and the α-tubulin spots) did not differ in the EpoB40 cells (Fig. 2C). This confirms the presence of the charge-altering mutation of βI-tubulin in EpoB40 cells (Fig. 2C and 2D) and supports the feasibility of the chosen proteomic workflow.

Differences in β-tubulin isotypes are an interesting source of candidate predictive markers for MIAs [1]. Tubulins constitute ~4% of total cellular protein [18] and are commonly detected in 2DE analysis of cells and tissue. However, individual β-tubulin isotypes have similar masses (~50 kDa) and isoelectrical points (pI) (~5.1) [18] and cannot be well resolved by conventional
2DE analysis. Relative expression of β-tubulin isotypes in different drug-resistant cell lines was determined by Western blot analysis (Fig. 2E). It has been reported that the βI-, βIII-, βIV- and βV-tubulin content in A549 cells was 50%, 8%, 36.5% and 5.5%, respectively, and those in Hey cells, 39.3%, 0.3%, 39.9% and 20.5%, respectively [19]. The level of βIII-tubulin was low in Hey cells, but increased by 8- and 15-fold in EpoB8 and Ixab80, respectively. This is consistent with the report that βIII-tubulin overexpression is related to drug resistance [1]. βIV-tubulin expression was decreased in both EpoB-resistant cell lines, EpoB40 and EpoB8. βV-tubulin expression was not altered in the A549 series, but was increased in both EpoB8 and Ixab80 cells.

**Actins, Keratins and Vimentin**

In the present study, actin was detected as a prominent string of spots by 2D DIGE and the spot farthest to the right was significantly increased only in AT12 cells and was found to represent either β- or γ- actin (not α-actin) (Fig. 3A and Table 1). We were not able to obtain sufficient MS-data to determine which of the two isoforms was increased in the AT12 cells (see Table S2). The β- and γ-actins coexist in most cell types whereas the α-actins are found in muscle tissues [20]. The actins have a similar mass (~42 kDa) and pI (~5.3) and high sequence homology. β- and γ-actin differ only in three N-terminal residues. We performed Western blotting for both β- and γ-actins using the pan-anti-β- and anti-γ-actin antibodies, but did not observe substantial changes in the total lysates of any of the resistant cell lines. The observed actin spot-pattern is reminiscent of a 2D DIGE study of a mouse xenograft model of acute lymphoblastic leukemia (ALL) [21]. In that study, the γ-actin spot was decreased in vincristine
resistant cells, whereas in our study the β/γ-actin spot was increased in AT12 cells. In the study of the mouse model of ALL, γ-actin was confirmed to be decreased by immuno-blotting and PCR, whereas β-actin was not changed [21]. Similar to actin, keratin was also present as a highly prominent string of spots and the keratin spot on the far right was also increased in AT12 cells (Fig. 3A and Table 1); however, the MS analysis could not discriminate between the ~20 epithelial keratin isoforms present in humans.

It has previously been suggested that actin could be implicated in resistance to MIAs [21]. The MT pellet from A549 cells was washed with a high salt solution to release MT-associated proteins and the wash solution was analyzed by 2DE. Interestingly, the most prominent protein spot was identified as β- or γ-actin actin (Fig. 3B and 3C), supporting a link between actin and tubulin and/or Taxol. As opposed to the actins, the abundant keratins were not detected in the salt wash fraction by 2DE.

Vimentin, an intermediate filament protein [22], was identified in two spots and both were increased only in Ixab80 cells according to 2D DIGE (Fig. 3D and Table 1). Vimentin was also increased in Ixab80 by Western blotting (Fig. 3E). We do not know the structural differences between the two isoforms of vimentin observed by 2D DIGE. A recent study concluded that two similar vimentin spots represent differential processing [23].

**Stathmin**

We previously reported that stathmin was slightly increased in AT12 cells and reduced in EpoB40 cells [14, 17]. In the present study, stathmin was detected as two distinct spots by 2D DIGE (Fig. 4A). The left stathmin spot (pI~ 5.5) was decreased in EpoB40, EpoB8 and Ixab80 cells.
(Fig. 4A and Table 1), whereas the right stathmin spot (pI~6.0) was not significantly changed in any of the drug resistant cells (Fig. 4A and Table 1). The A549 cells were analyzed by 2DE and phospho-specific staining and the results supported the phosphorylation of both stathmin spots (Fig. 4B). In a 2D DIGE study of mouse liver progenitor-29 cells [24], two similar spots were also found to represent phosphorylated stathmin by using antibodies against stathmin phosphosites [24]. Stathmin can be phosphorylated at serines 16, 25, 38 and 63 and this can change both the isoelectrical point and the electrophoretic mobility of the protein. Mammalian stathmin has been identified in at least 14 unique 2DE spots representing two unphosphorylated and 12 phosphorylated molecular variants that are all present within a relatively small area of the 2D gel (pI 5.5 - 6.2 & MW 19 - 23 kDa) [25]. Western blotting using antibodies for total stathmin demonstrated similar results as reported previously for EpoB40 and AT12 cells [14, 17] and showed similar levels of stathmin in sensitive and resistant Hey cells, whereas Western blotting with an antibody specific for S16-P-stathmin revealed a significant increase in the AT12 and the Ixab80 cell lines (Fig. 4C). We also demonstrated that in the Taxol-resistant breast cancer cell line K20T [26], total stathmin levels were decreased, but S16-phospho-stathmin levels were markedly increased, compared to the drug sensitive cell line MDA-MB-231 (Fig. S1A). As such, our observations support the idea that differential phosphorylation of stathmin could have a role in resistance to MIAs.

Cytoskeleton-associated proteins: 14-3-3σ, Galectin-1, CLIC1, Ezrin, and NM23-H1

We identified five proteins that have been associated with both the cytoskeleton and with cancer, including 14-3-3σ, galectin-1, chloride intracellular channel protein 1 (CLIC1), ezrin
and the metastasis inhibitor factor nm23-H1 (Table 1). Among these five candidate cytoskeleton-associated proteins that we identified, we focused on two proteins, 14-3-3σ and galectin 1, and studied their role in drug resistance, since their expression was increased markedly in the resistant cells (Fig. 5A). In the present study 14-3-3σ was increased dramatically only in AT12 cells (Table 1) and this was confirmed by Western blotting, although 14-3-3σ was also moderately increased in EpoB40 and EpoB8 cells (Fig. 5A). According to 2D DIGE, galectin-1 was strongly increased only in EpoB40 cells and moderately increased in EpoB8 and Ixab80 (Table 1). This was supported by Western blotting (Fig. 5A). Galectin-1 expression was also moderately increased in the Taxol-resistant breast cancer cell line K20T (Fig. S1B). CLIC1, ezrin and nm23-H1 were differentially expressed in different drug resistant cells (see Fig. S2).

The effect of galectin-1 suppression on drug sensitivity was examined by transfecting cells with galectin-1 siRNA followed by cytotoxicity studies. Transfection of cells with galectin-1 siRNA suppressed the expression of galectin-1 in all cell lines tested, and this suppression lasted for at least 6-7 days (Fig. S3). Cytotoxicity assays indicated that suppression of galectin-1 in Hey and EpoB8 cells increased EpoB sensitivity by 58.3% and 57.7%, respectively, as determined by IC50 values (Fig. 5B). Similar results were obtained for Ixab80 cells (Fig. S4). In contrast, galectin-1 suppression did not cause an increase in cisplatin sensitivity in either Hey or EpoB8 cells (data not shown). Interestingly, galectin-1 levels also were higher in the growth medium from EpoB8 and Ixab80, compared to that of the drug-sensitive Hey cells (Fig. 5C). However, galectin-1 siRNA only caused a minimal effect on EpoB sensitivity in EpoB40 cells and this may be related to the high resistance of the cell line, most likely associated with the β292 mutation [13].
Although 14-3-3σ expression was markedly increased in AT12 cells, suppression of 14-3-3σ expression by siRNA did not result in drug sensitivity changes (data not shown).

Finally, we also observed differences in several heat shock proteins (HSPs), including HSP90, GRP78 and HSP27, as well as carbonic anhydrase 2 (CA2) (Table 1). These proteins are likely unspecific markers of cellular stress [12].

**Discussion**

Cell lines resistant to the MSAs, Taxol, epothilone B and ixabepilone were analyzed to identify proteomic changes associated with *in vitro* drug resistance. The chosen strategy of comparing several drug resistant cell lines in parallel was taken with the expectation of identifying drug-specific proteomic changes of significant clinical interest, as opposed to unspecific/stress-induced changes. In particular, we propose that differential abundance of galectin-1 and 14-3-3σ, as well as differential phosphorylation of stathmin, are worthy of further study in tumors resistant to MSAs and as candidate predictive biomarkers for MSAs.

Galectin-1 protein was significantly increased in the EpoB-resistant cell lines EpoB40 and EpoB8, and in the ixabepilone resistant cell line Ixab80. Suppression of galectin-1 increased drug sensitivity, suggesting that galectin-1 may play a role in drug resistance (see Fig. 5). Although galectin-1 was not increased in AT12, it was found to be increased in a Taxol-resistant breast cancer cell line K20T [26] (Fig. S3B). Galectin-1 belongs to a family of carbohydrate-binding proteins with an affinity for β-galactosides. It has been reported that galectin-glycan
lattices regulate cell surface glycoprotein organization and signaling [27]. In a previous study, galectin-1 promoted tumor invasion, in part, by regulating the actin cytoskeleton [28], and it has been suggested that inhibition of galectin-1 expression could be a therapeutic target in cancer [29]. Recently, several findings support this hypothesis: (a) high galectin-1 expression correlated with poor prognosis in epithelial ovarian cancer [30]; (b) galectin-1 expression was associated with tumor invasion and metastasis in stage IB to IIA cervical cancer [31]; (c) galectin-1 was significantly up-regulated during prostate cancer progression. Silencing of galectin-1 in prostate cancer cells reduced tumor vascularization, therefore, it has been suggested that galectin-1 could serve as a target for anti-angiogenic therapy in advanced stages of prostate cancer [32]. In addition, it has been demonstrated that p38MAPK, ERK and cyclooxygenase-2 participate in galectin-1 mediated tumor progression and chemoresistance to cisplatin in lung cancer [33]. Fifteen mammalian galectins have been identified; serum galectin-2, -4, and -8 are markedly increased in colon and breast cancer patients [34]. In our study, we found that galectin-1 levels in the growth medium were increased in drug-resistant ovarian cancer cells, compared to the drug-sensitive cells, (Fig. 5C) suggesting that galectin-1 may serve as a marker for drug resistance.

We also found that 14-3-3σ was significantly increased in the Taxol-resistant cell line AT12 and minimally in the epothilone/ixabepilone resistant cell lines. 14-3-3σ is believed to play a role in regulating cytoskeletal dynamics [35]. However, suppression of 14-3-3σ by siRNA transfection did not alter drug sensitivity (data not shown). Treatment with the MIA vincristine has previously been associated with both up and down-regulation of individual 14-3-3 protein isoforms (there are seven 14-3-3 genes in humans; 14-3-3 alpha to zeta) and also modified
versions of 14-3-3 isoforms [36]. Differential expression of 14-3-3σ is associated with tumor progression and drug-resistance [10].

Our results also support the idea that differential phosphorylation of stathmin is associated with resistance to Taxol. Increased levels of total stathmin have previously been associated with resistance to both Taxol and vinblastine [1], and also with resistance to other classes of cancer drugs [10]. Differential phosphorylation of stathmin has previously been implicated in prostate cancer progression [37]; however, differential phosphorylation of stathmin in resistance to MIAs has not been well documented. Dephosphorylation of stathmin induces binding of stathmin to MTs and promotes disassembly of MTs. Thus, an intriguing possibility is that differential phosphorylation of stathmin promotes cellular resistance by counteracting the effect of the MIAs on the MTs. In particular, we propose that further studies should examine phosphorylation of stathmin serine 16 as a candidate marker for resistance to ixabepilone in ovarian cancer cells.

Among the proteins identified (see Table 1 and Fig. 2), tubulin, especially βIII-tubulin, has previously been reported to be associated with drug resistance [1]. Overexpression of mouse βV-tubulin has been shown to confer Taxol resistance [38]. Aberrant expression of βV-tubulin in breast, lung and ovarian cancers also suggest that βV-tubulin may be associated with tumorigenesis [39]. Hey cells express high levels of βV-tubulin (20%), compared to other eukaryotic sources, and our drug-resistant Hey cells overexpress βV-tubulin, further suggesting that human βV-tubulin may be important in MSA resistance.
In agreement with previous observations [21], we provide evidence that actin could have a role in resistance to MIAs. Both actin and the MT cytoskeleton are responsible for fundamental cellular processes and are both strongly implicated in carcinogenesis [20]. Previous proteomic studies have implicated actin in resistance to MIAs [1]. A recent report also demonstrated that partial depletion of γ-actin suppressed MT dynamics, delayed mitotic progression and inhibited cell proliferation [40]. Our study adds to the evidence that β- and/or γ-actin, or specific isoforms hereof, could be associated with resistance to MSAs.

We also identified changes in other proteins associated with the cytoskeleton, including vimentin, CLIC1, ezrin and nm23-H1. Vimentin is a prominent intermediate filament protein. Taxol has been shown to induce hyperphosphorylation of vimentin and cause reorganization of the vimentin intermediate filament structure [41]. Vimentin was shown to be increased in leukemic cell lines selected for resistance to vincristine [36]. CLIC1 is increased in several cancers [42] and it has been proposed that the family of CLICs interact with the actin cytoskeleton [43]. Ezrin is also implicated in cancer and metastasis [44] and in regulation of the actin cytoskeleton [45]. NM23 belongs to the class of metastasis suppressor genes [46], and expression of the NM23 affects tubulin polymerization [47]. Combined, these results suggest that the mechanism responsible for resistance to MSAs may include many proteins associated with the cytoskeleton.

An interesting observation deserves mentioning. In a previous study, CLIC1 and ezrin were decreased in vincristine resistant cancers [21], whereas in this study CLIC1 was increased in Taxol and EpoB resistant cells, and ezrin was increased in EpoB40 cells. Similarly, the right-
most actin spot was decreased in vincristine resistant cells [21], whereas in our study the right-most spot was increased in AT12 cells (see Fig. 3A). This could suggest that the direction of change of some proteins is related to the mechanism of action of the drug; - vincristine is an MT-destabilizer, whereas Taxol and EpoB are both MT-stabilizers, although both vincristine and Taxol alter microtubule dynamicity at low concentrations. There are, however, many instances of proteins being both up and down regulated in different studies of drug resistance (e.g. HSP27 [10]) and so the direction of change may also be related to the exact experimental details.

It is well accepted that the development of drug resistance is a multifactorial process. It has been reported that Taxol influences the interaction between MTs and the MT binding domain of MAP4 [48]. Taxol-mediated changes in MAP binding to MTs may affect the downstream signaling pathways in cells. Therefore, resistant cells selected with MSAs may have altered expression of many cellular proteins. Several factors may complicate the analysis of in vitro drug resistance: (a) the resistant cell lines were derived from cancer cell lines of different origins, and hence with different tissue context; (b) the four resistant cell lines used did not exhibit a similar fold of resistance; (c) different resistant cell lines harbor different tubulin mutations, some of which may alter drug binding while others may influence the interaction between tubulin and endogenous regulatory proteins; (d) some resistant cells, such as EpoB8, have no tubulin mutations, therefore, different proteins might be induced to confer a drug resistance phenotype. It has been suggested that every presently known drug has approximately six target proteins [49]. In addition, although differences in the level of post-translational modifications of proteins can be implicated in drug resistance [50], 2DE-based proteomics is biased towards the detection of more abundant proteins, such as structural
proteins. Drug resistant cells may harbor proteomic changes below the limit of detection with 2DE.

In summary, our results indicate that although the resistant cells were selected with MSAs, the expression of many cytoskeletal associated proteins was altered, confirming that there is a close link between the MT system and other cytoskeletal components. The present study identifies several candidate proteomic changes that may play a role in resistance to MIAs and deserves further analysis in human tumor samples. Specifically, the results suggest that several cytoskeleton-associated proteins, particularly galectin-1, should be further studied in vivo to determine if they could be developed as predictive biomarkers for drug resistance.

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### Table 1

Identification of proteins differentially expressed in drug-resistant cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Code</th>
<th>A549 cell series</th>
<th>Hey cell series</th>
</tr>
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<tbody>
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<td><strong>Chloride Intracellular Channel</strong></td>
<td>CLIC1</td>
<td>3.2 ± 0.4 b</td>
<td>- (1.8 ± 0.4)</td>
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<tr>
<td><strong>Protein 1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Stathmin (right spot, pI 6.0)</td>
<td>STMN1</td>
<td>-</td>
<td>- (2.4 ± 0.5) c</td>
</tr>
<tr>
<td>Stathmin (left spot, pI 5.5)</td>
<td>STMN1</td>
<td>-</td>
<td>2.3 ± 1.2</td>
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<tr>
<td><strong>Beta-/Gamma-Actin</strong></td>
<td>ACTB/G</td>
<td>3.5 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td><strong>Beta 1-Tubulin (Gln to Glu)</strong></td>
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<td><strong>Keratin</strong></td>
<td>KRT</td>
<td>-</td>
<td>2.0 ± 0.6</td>
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<tr>
<td><strong>Vimentin</strong></td>
<td>VIME</td>
<td>-</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>14-3-3 Sigma</td>
<td>1433S</td>
<td>-</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td><strong>Ezrin</strong></td>
<td>EZR1</td>
<td>2.1 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Galectin-1</strong></td>
<td>LEG1</td>
<td>2.3 ± 0.3</td>
<td>- (1.9 ± 0.4)</td>
</tr>
<tr>
<td><strong>Metastasis Inhibition Factor NM23</strong></td>
<td>NM23H1</td>
<td>-</td>
<td>- (2.1 ± 0.5)</td>
</tr>
<tr>
<td><strong>Heat Shock Protein 90</strong></td>
<td>HSP90</td>
<td>2.3 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td><strong>Glucose-Regulated Protein 78</strong></td>
<td>GRP78</td>
<td>2.0 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td><strong>Heat Shock Protein 27</strong></td>
<td>HSP27</td>
<td>2.7 ± 1.2</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td><strong>Carbonic Anhydrase 2</strong></td>
<td>CA2</td>
<td>- (3.1 ± 1.5)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data were obtained after 2D DIGE coupled with MALDI TOF/TOF mass spectrometry analysis.

* Fold-change: expression level of proteins in the drug-resistant cells compared to the drug-sensitive cells. Those numbers not in bold have a mean fold-change of less than 2.

* Mean ± SD, n=3.

* “Minus” values represent fold-decrease in protein expression.
Figure legends

Figure 1. Experimental design for proteomics of cancer cell lines resistant to MSAs studied by 2D DIGE and mass spectrometry. The study includes a total of six cell lines that were analyzed in triplicate on a total of nine gels. Each gel contained two samples and a pooled standard.

Figure 2. Alterations in tubulin isotype expression in drug-resistant cells. A charge-altering mutation in βI-tubulin was detected by 2D DIGE (A to D). (A) Silver stained 2D gel of A549 total lysates. (B) Silver stained 2DE image of tubulin-depleted A549 lysates for improved resolution of α- and β-tubulin. (C) 2D DIGE analysis of tubulin-depleted fractions of A549 (red) and EpoB40 (green). The arrows (1 and 2) indicate two spots that are increased in EpoB40. (D) Resolution of tubulin isotypes by isoelectrofocusing, adapted from Yang et al., MCT 4:987, 2005. (C and D) 1, mono-glutamylated mutant βI-tubulin; 2, mutant βI-tubulin; 3, wild type βI-tubulin. (E) Expression levels of βI-, βIII-, βIV- and βV-tubulin in the total lysates of drug sensitive and resistant cells were determined by Western blot analyses using isotype-specific anti-tubulin antibodies (Table S1). Polyclonal human anti-βV-tubulin antibody was developed in this laboratory (37). Lower panel: Ponceau-stained blot showing equal protein loading.

Figure 3. Expression of actins, keratins and vimentin in drug-resistant cell lines. See Fig. 1 for proteomic changes in drug-resistant cell lines in the high mass range (30 to 80 kDa). (A) Silver stained keratins and actins in 2D DIGE of A549 and AT12 cells; two spots representing
significantly increased actin and keratin in AT12 cells. (B) Silver stained 2D gel of A549 total lysates. (C) Silver stained 2DE image of proteins released from a MT pellet by salt wash. (D) Silver stained vimentin in 2D DIGE of Hey and Ixab80 cells. (E) Expression of vimentin in the total lysate of drug-sensitive and -resistant cells was determined by Western blot analysis.

Figure 4. Expression and phosphorylation of stathmin in drug-resistant cell lines. See Fig. 1 for proteomic changes in drug-resistant cell lines in the low mass range (10 to 35 kDa). (A) Selected 2D DIGE region showing two spots identified as stathmin 1 (STMN1). (B) The same region after phospho-specific stain. (C) Expression of stathmin and Ser16-Phospho-stathmin in the total lysates of drug-sensitive and -resistant cells was determined by Western blot analysis.

Figure 5. Expression of 14-3-3σ and galectin-1 was increased differentially in drug-resistant cells and suppression of galectin-1 expression increased drug sensitivity in both drug-sensitive and -resistant Hey cells. (A) Expression of 14-3-3σ and galectin-1 in the total cell lysates was determined by Western blotting. (B) Hey and EpoB8 cells were transfected with vehicle only (C, dotted lines), 30 nM galectin-1 siRNA (solid lines), or 30 nM non-targeting siRNA (NT, dashed lines), followed by SRB cytotoxicity assays after 72 h. IC$_{50}$ values for vehicle only and for galectin-1 siRNA transfection are labeled. The statistical significance of experimental data were evaluated using student’s t test where * and ** indicate that results were statistically significant at $P<0.05$ and $P<0.01$, respectively. (C) Galectin-1 level in the growth medium of Hey, EpoB8 and Ixab80 cells. Lower panel: proteins in the growth medium showing equal loading.
Figure 2
Figure 3
Figure 4
**Figure 5**

(A) Western blot analysis of 14-3-3σ and Galectin-1 in A549 and Hey series. Fold Change: A549 series (1, 7.1, 38) and Hey series (1, 1.4, 0.9) for 14-3-3σ, and (1, 15.1, 2.3) and (1, 2.5, 1.8) for Galectin-1.

(B) Western blot analysis of Galectin-1 and α-tubulin in Hey and EpoB8 series. C (100%, NT (non-treated) for Hey, and C (100%, siRNA, NT for EpoB8. Fold Change: Hey (1, 0, 71) and EpoB8 (100, 31, 115) for Galectin-1, and Hey (1, 0, 71) and EpoB8 (100, 31, 115) for α-tubulin.

(C) Western blot analysis of Galectin-1 in Hey, Epo8, and Ixab80 series. Fold Increase: Hey (1), Epo8 (43), Ixab80 (22).

Graphs showing the effect of EpoB (nM) on percent growth in Hey and EpoB8 cells at different concentrations: 0.24 nM (Hey, EpoB8), 0.38 nM (Hey), 2.6 nM (Hey), 4.1 nM (EpoB8).
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Proteomics of cancer cell lines resistant to microtubule stabilizing agents

Jakob Albrethsen, Ruth H. Angeletti, Susan Band Horwitz, et al.

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