Proteomics of Cancer Cell Lines Resistant to Microtubule-Stabilizing Agents

Jakob Albrethsen¹, Ruth H. Angeletti¹, Susan Band Horwitz², and Chia-Ping Huang Yang²,³

Abstract

Despite the clinical success of microtubule-interacting agents (MIA), a significant challenge for oncologists is the inability to predict the response of individual patients with cancer 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 (MSA). The human lung cancer cell line A549 was compared with 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 with two drug-resistant daughter cell lines, an EpoB-resistant cell line (EpoB88) 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 14-3-3e, 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 MSAs. Mol Cancer Ther; 13(1); 260–9. ©2013 AACR.

Introduction

The microtubule-interacting agents (MIA) are a successful class of cancer drugs (1, 2). Several MIAs have been clinically approved and others are under development (1–3). Despite 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 function and thereby disrupt cell division and induce cell death (1). The targets of the MIAs are either the microtubules themselves or the free heterodimers made up of α- and β-tubulins. Differential expression of tubulin isotypes, tubulin mutations, and posttranslational 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 microtubule-associated proteins (MAP) 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 microtubules 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 are 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 in vitro resistance to MIAs, specifically, to 3 microtubule-stabilizing agents (MSA), 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 6 selected cell lines was conducted. Our study includes 1 cell line resistant to taxol, 2 cell lines resistant to EpoB, and 1 cell line resistant to the EpoB-derivative ixabepilone, as well as 2 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% FBS. A549 was obtained from American Type Culture Collection in 1990 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 nmol/L taxol, 40 nmol/L thio-urea, 4% CHAPS, pH 8.5). Samples were labeled with Cy3 dye (GE) as described (12).Isolectric focusing was done with 24 cm pH 4–10 nonlinear IPG strips on the Ettan IPGhorII (GE), and the second dimension separation was conducted on 13% SDS-PAGE gels (JULE Biotechnology). The experiment was carried out in 2 batches of 3 gels; one batch for the A549 cells and one for the Hey cells (see Fig. 1). Each gel included 2 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 conducted as described for the total lysates and this experiment included 9 samples, including 5 standards, and was run on 3 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 2 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 mmol/L 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 were determined by absorption assay (GE). Before 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 mmol/L Tris, 7 mol/L urea, 2 mol/L thio-urea, 4% CHAPS, pH 8.5). Samples were labeled with Cy3 dye (GE) as described (12). Isoelectric focusing was done with 24 cm pH 4–10 nonlinear IPG strips on the Ettan IPGhorII (GE), and the second dimension separation was conducted on 13% SDS-PAGE gels (JULE Biotechnology). The experiment was carried out in 2 batches of 3 gels; one batch for the A549 cells and one for the Hey cells (see Fig. 1). Each gel included 2 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 conducted as described for the total lysates and this experiment included 9 samples, including 5 standards, and was run on 3 gels in one independent batch.

Suppression of galectin-1 expression by RNA interference and cytotoxicity

Cells were transfected for 24 hours with 30 nmol/L galectin-1 siRNA (Santa Cruz) and 30 nmol/L non-targeting
siRNA (Thermo Scientific) using the transfection reagent Lipofectamine RNAiMAX (Invitrogen). Transfected cells were replated in 96-well culture dishes at 1,000 to 2000 cells per well and treated with increasing concentrations of drugs. Sulforhodamine B (SRB) cytotoxicity assays were conducted after 72 to 96 hours. 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 with 2 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 with 2 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- 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 β-I-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 nmol/L 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 β-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 with 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 and P < 0.05). We present and validate the proteomic changes identified by 2D DIGE with particular focus on candidate predictive biomarkers (Table 1 and Fig. 1; Supplementary Table S2).

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 B): mammalian α-tubulin has a slower electrophoretic mobility than β-tubulin and the average theoretical pls 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 2 leftmost β-tubulin spots were significantly increased (>2.0-fold and 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 β-tubulin in EpoB40 cells (Fig. 2C and D) and supports the feasibility of the chosen proteomic workflow.

Differences in β-tubulin isotypes are an interesting source of candidate predictive markers for MIs (1). Tubulins constitute about 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; ref. 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-, βV-, and βV'-tubulin content in A549 cells was 50%, 8%, 36%, and 5.5%, respectively, and those in Hey cells was 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 2 isoforms was increased in the AT12 cells (see Supplementary 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-actins differ only in 3 N-terminal residues. We conducted 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; ref. 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 immunoblotting 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Code</th>
<th>EpoB40</th>
<th>AT12</th>
<th>EpoB8</th>
<th>Ixab80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride intracellular channel protein 1</td>
<td>CLIC1</td>
<td>3.2 ± 0.4</td>
<td>—</td>
<td>—</td>
<td>(1.8 ± 0.4)</td>
</tr>
<tr>
<td>Stathmin (right spot, pI 6.0)</td>
<td>STMN1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Stathmin (left spot, pI 5.5)</td>
<td>STMN1</td>
<td>(2.4 ± 0.5)</td>
<td>—</td>
<td>—</td>
<td>(1.5 ± 0.4)</td>
</tr>
<tr>
<td>β-γ-Actin</td>
<td>ACTB/G</td>
<td>—</td>
<td>2.3 ± 1.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β1-Tubulin (Gln to Glu)</td>
<td>TBB</td>
<td>3.5 ± 0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Keratin</td>
<td>KRT</td>
<td>—</td>
<td>2.2 ± 0.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vimentin</td>
<td>VIME</td>
<td>—</td>
<td>—</td>
<td>2.0 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>14-3-3 σ</td>
<td>1433S</td>
<td>—</td>
<td>2.1 ± 0.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ezrin</td>
<td>EZR1</td>
<td>2.1 ± 0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Galectin-1</td>
<td>LEG1</td>
<td>2.3 ± 0.3</td>
<td>—</td>
<td>1.9 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Metastasis inhibition factor NM23</td>
<td>NM23H1</td>
<td>—</td>
<td>—</td>
<td>(2.1 ± 0.5)</td>
<td>(2.0 ± 0.2)</td>
</tr>
<tr>
<td>Heat shock protein HSP90</td>
<td>HSP90</td>
<td>2.3 ± 0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glucose-regulated protein 78</td>
<td>GRP78</td>
<td>2.0 ± 0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heat shock protein HSP27</td>
<td>HSP27</td>
<td>2.7 ± 1.2</td>
<td>—</td>
<td>1.9 ± 0.7</td>
<td>—</td>
</tr>
<tr>
<td>Carbonic anhydrase 2</td>
<td>CA2</td>
<td>(3.1 ± 1.5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

**Table 1. Identification of proteins differentially expressed in drug-resistant cells**

**Fold change**

<table>
<thead>
<tr>
<th></th>
<th>A549 cell series</th>
<th>Hey cell series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EpoB40</td>
<td>AT12</td>
</tr>
<tr>
<td>Chloride intracellular channel protein 1</td>
<td>CLIC1</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Stathmin (right spot, pI 6.0)</td>
<td>STMN1</td>
<td>—</td>
</tr>
<tr>
<td>Stathmin (left spot, pI 5.5)</td>
<td>STMN1</td>
<td>(2.4 ± 0.5)</td>
</tr>
<tr>
<td>β-γ-Actin</td>
<td>ACTB/G</td>
<td>—</td>
</tr>
<tr>
<td>β1-Tubulin (Gln to Glu)</td>
<td>TBB</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>Keratin</td>
<td>KRT</td>
<td>—</td>
</tr>
<tr>
<td>Vimentin</td>
<td>VIME</td>
<td>—</td>
</tr>
<tr>
<td>14-3-3 σ</td>
<td>1433S</td>
<td>—</td>
</tr>
<tr>
<td>Ezrin</td>
<td>EZR1</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Galectin-1</td>
<td>LEG1</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Metastasis inhibition factor NM23</td>
<td>NM23H1</td>
<td>—</td>
</tr>
<tr>
<td>Heat shock protein HSP90</td>
<td>HSP90</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Glucose-regulated protein 78</td>
<td>GRP78</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Heat shock protein HSP27</td>
<td>HSP27</td>
<td>2.7 ± 1.2</td>
</tr>
<tr>
<td>Carbonic anhydrase 2</td>
<td>CA2</td>
<td>(3.1 ± 1.5)</td>
</tr>
</tbody>
</table>

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

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

**Mean ± SD, n = 3.**

**c**| Minus values represent fold decrease in protein expression.

www.aacrjournals.org Mol Cancer Ther; 13(1) January 2014 263
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 microtubule pellet from A549 cells was washed with a high salt solution to release MAPs 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 C), 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 2 spots and both were increased only in drug-resistant cells. 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 (30–80 kDa). A, silver-stained keratins and actins in 2D DIGE of A549 and AT12 cells; 2 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 microtubule 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.
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 2 isoforms of vimentin observed by 2D DIGE. A recent study concluded that 2 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 2 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), 2 similar spots were also found to represent phosphorylated stathmin by using antibodies against stathmin phospho-sites (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 2 unphosphorylated and 12 phosphorylated molecular variants that are all present within a relatively small area of the 2D gel (pI, 5.5–6.2 and MW, 19–23 kDa; ref. 25). Western blotting using antibodies for total stathmin showed 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-phospho-stathmin revealed a significant increase in the AT12 and the Ixab80 cell lines (Fig. 4C). We also showed 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 with the drug-sensitive cell line MDA-MB-231 (Supplementary 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: 14-3-3s, galectin-1, CLIC1, ezrin, and NM23-H1**

We identified 5 proteins that have been associated with both the cytoskeleton and with cancer, including 14-3-3s, galectin-1, chloride intracellular channel protein 1 (CLIC1), ezrin, and the metastasis inhibitor factor nm23-H1 (Table 1). Among these 5 candidate cytoskeleton-associated proteins that we identified, we focused on 2 proteins, 14-3-3s and galectin-1, and studied their role in drug resistance, as their expression was increased markedly in the resistant cells (Fig. 5A). In the present study, 14-3-3s was increased dramatically only in AT12 cells (Table 1) and this was confirmed by Western blotting, although 14-3-3s 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 (Supplementary Fig. S1B). CLIC1, ezrin, and nm23-H1 were differentially expressed in different drug-resistant cells (see Supplementary 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 to 7 days (Supplementary 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 (Supplementary 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 with 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 (HSP), 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 14-3-3s, 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 (ref. 26; Supplementary Fig. S1B). 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: (i) high galectin-1 expression correlated with poor prognosis in epithelial ovarian cancer (30); (ii) galectin-1 expression was associated with tumor invasion and metastasis in stage IB to IIA cervical cancer (31); and (iii) galectin-1 was significantly upregulated 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 antiangiogenic therapy in advanced stages of prostate cancer (32). In addition, it has been shown that p38MAPK, extracellular signal-regulated kinase (ERK), and COX-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 patients with colon and breast cancer (34). In our study, we found that galectin-1 levels in the growth medium were increased in drug-resistant ovarian cancer cells, compared with the drug-sensitive cells (Fig. 5C), suggesting that galectin-1 may serve as a marker for drug resistance.

We also found that 14-3-3s was significantly increased in the taxol-resistant cell line AT12 and minimally in the epothilone/ixabepilone-resistant cell lines. 14-3-3s is believed to play a role in regulating cytoskeletal dynamics (35). However, suppression of 14-3-3s by siRNA transfection did not alter drug sensitivity (data not shown). Treatment with the MIA vincristine has previously been associated with both up- and downregulation 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 14-3-3s 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 microtubules and promotes disassembly of microtubules. Thus, an intriguing possibility is that differential phosphorylation of stathmin promotes cellular resistance by counteracting the effect of the MIAs on the microtubules. 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 with other eukaryotic sources, and our drug-resistant Hey cells overexpress βV-tubulin, further suggesting that human βV-tubulin may be important in MSA resistance. 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 rightmost actin spot was decreased in vincristine-resistant cells (21), whereas in our study, the rightmost 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 a microtubule destabilizer, whereas taxol and EpoB are both microtubule stabilizers, although both vincristine and taxol alter microtubule dynamics at low concentrations. There are, however, many instances of proteins being both up- and downregulated in different studies of drug resistance (e.g., HSP27; ref. 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 microtubules and the microtubule-binding domain of MAP4 (48). Taxol-mediated changes in MAP binding to microtubules 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: (i) the resistant cell lines were derived from cancer cell lines of different origins, and hence with different tissue context; (ii) the 4 resistant cell lines used did not exhibit a similar fold of resistance; (iii) different resistant cell lines harbor different tubulin mutations, some of which may alter drug binding whereas others may influence the interaction between tubulin and endogenous regulatory proteins; and (iv) 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 6 target proteins (49). In addition, although differences in the level of posttranslational modifications of proteins can be implicated in drug resistance (50), 2DE-based proteomics is biased toward 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 microtubule system and other cytoskeletal components. The present study identifies several candidate proteomic changes that may play a role in resistance to MIA 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 whether they could be developed as predictive biomarkers for drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Albrethsen, R.H. Angeletti, S.B. Horwitz, C.-P.H. Yang
Development of methodology: R.H. Angeletti
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Albrethsen, R.H. Angeletti, C.-P.H. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Albrethsen, R.H. Angeletti, S.B. Horwitz, C.-P.H. Yang
Writing, review, and/or revision of the manuscript: J. Albrethsen, R.H. Angeletti, S.B. Horwitz, C.-P.H. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.H. Angeletti
Study supervision: S.B. Horwitz, C.-P.H. Yang

Acknowledgments

The authors thank Jurriaan Brouwer and Dr. Gloria Huang for assistance with cell line STR profiling.

Grant Support

This work was supported by NIH CA077263 (S.B. Horwitz, H. McDaid), the National Foundation for Cancer Research (S.B. Horwitz), the Breast Cancer Research Foundation (S.B. Horwitz, H. McDaid), and NIH CA101150 (R.H. Angeletti).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 11, 2013; revised November 4, 2013; accepted November 6, 2013; published OnlineFirst November 19, 2013.

References


www.aacrjournals.org Mol Cancer Ther; 13(1) January 2014 269

Published OnlineFirst November 19, 2013; DOI: 10.1158/1535-7163.MCT-13-0471

Downloaded from mct.aacrjournals.org on June 20, 2017. © 2014 American Association for Cancer Research.
Molecular Cancer Therapeutics

Proteomics of Cancer Cell Lines Resistant to Microtubule-Stabilizing Agents

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


Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0471

Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2014/01/15/1535-7163.MCT-13-0471.DC1

This article cites 49 articles, 14 of which you can access for free at: http://mct.aacrjournals.org/content/13/1/260.full.html#ref-list-1

This article has been cited by 2 HighWire-hosted articles. Access the articles at: /content/13/1/260.full.html#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.