Mechanisms of Resistance to Cabazitaxel

George E. Duran, Yan C. Wang, E. Brian Francisco, John C. Rose, Francisco J. Martinez, John C. Coller, Diana Brassard, Patricia Vrignaud, and Branimir I. Sikic

Abstract

We studied mechanisms of resistance to the novel taxane cabazitaxel in established cellular models of taxane resistance. We also developed cabazitaxel-resistant variants from MCF-7 breast cancer cells by stepwise selection in drug alone (MCF-7/CTAX) or drug plus the transport inhibitor PSC-833 (MCF-7/CTAX-P). Among multidrug-resistant (MDR) variants, cabazitaxel was relatively less cross-resistant than paclitaxel and docetaxel (15- vs. 200-fold in MES-SA/Dx5 and 9- vs. 60-fold in MCF-7/TTx50, respectively). MCF-7/TTx50 cells that were negative for MDR but had 9-fold resistance to paclitaxel were also 9-fold resistant to cabazitaxel. Selection with cabazitaxel alone (MCF-7/CTAX) yielded 33-fold resistance to cabazitaxel, 52-fold resistance to taxanes (cabazitaxel or docetaxel) relative to MCF-7 parental cells. Class III β-tubulin (TUBB3) RNA and protein were elevated in both MCF-7/CTAX and MCF-7/CTAX-P. Decreased BRCA1 and 3-fold residual resistance to cabazitaxel with MDR inhibition. The MCF-7/CTAX-P variant did not express ABCB1, nor did it efflux rhodamine-123, BODIPY-labeled paclitaxel, and [3H]-docetaxel. These cells are hypersensitive to depolymerizing agents (vinca alkaloids and colchicine), have reduced baseline levels of stabilized microtubules, and impaired tubulin polymerization in response to taxanes (cabazitaxel or docetaxel) relative to MCF-7 parental cells.

Introduction

The taxanes paclitaxel (Taxol) and docetaxel (Taxotere) have substantial clinical activity in breast, ovarian, lung, and other cancers. These tubulin-active agents stabilize microtubules, blocking cells in the late G2-M phase of the cell cycle, resulting in cell death (1). Their clinical efficacy is limited by preexisting or acquired drug resistance. We have previously derived multiple paclitaxel- and docetaxel-selected variants in human breast and ovarian cancer cell lines. In these studies, selection with taxanes alone induced multidrug resistance (MDR) related to expression of P-glycoprotein (P-gp; ref. 2), and this resistance can be modulated in the presence of known transport inhibitors. Incorporation of the potent P-gp inhibitor PSC-833 during taxane selection has enabled us to establish non-MDR cellular models of taxane resistance. The taxane resistance observed in these variants is not associated with alterations in drug transport, but may include alterations in tubulin expression, apoptotic proteins, and cell-cycle regulation (3–6).

Several semisynthetic taxane analogues have been developed with the goal of evading drug resistance, including cabazitaxel (Jevtana, XRP6258), which was selected for clinical development based on its in vivo activity in docetaxel-resistant MDR tumor models. Preclinical studies indicated that this taxane is as potent as docetaxel in cellular models, and more effective in variants selected for resistance to taxanes (7). In 2010, the FDA-approved cabazitaxel in combination with prednisone/prednisolone for the treatment of patients with metastatic hormone-refractory prostate cancer previously treated with docetaxel (8–10).

This study assessed cabazitaxel activity and resistance mechanisms in several taxane-resistant variants, as well as two new cabazitaxel-selected variants of MCF-7 breast cancer cells, one selected with cabazitaxel alone and another coselected with PSC-833.

Materials and Methods

Drugs and reagents

The anticancer drugs cisplatin, colchicine, daunorubicin, doxorubicin, paclitaxel, vinblastine, and vincristine were obtained from the drug repository of the National Cancer Institute (Bethesda, MD). Docetaxel and cabazitaxel (XRP6258, Jevtana, formerly RPR116258A; Supplementary Fig. S1A) were gifts from Sanofi Oncology. Novartis Pharmaceuticals kindly provided the P-gp inhibitor PSC-833 (valspodar). All drugs were prepared in 100% ethanol as 1 mmol/L stock solutions and stored at −20°C. All other chemicals were purchased from the Sigma-Aldrich Chemical Co.
Cell culture and establishment of cabazitaxel-resistant MCF-7 variants

The MCF-7 human breast adenocarcinoma and OVCAR-3 human ovarian adenocarcinoma cell lines were purchased from the ATCC (purchased 6/1999). The human ovarian clear cell carcinoma cell line ES-2, human ovarian carcinoma MES-OV, and the human uterine sarcoma cell line MES-SA were established in our laboratory (MES-SA and ES-2 authenticated and submitted to the ATCC as CRL-1976 and -1978, respectively, and the MES-OV was submitted to the ATCC 7/2014). Cells were grown in McCoy 5A medium supplemented with 10% (v/v) FCS, 100 U of penicillin/mL, and 100 μg/mL streptomycin/mL (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO₂. Recombinant human IFN (CSL Behring) was added at a concentration of 1000 U/mL on April 30, 2017. © 2015 American Association for Cancer Research. mct.aacrjournals.org Downloaded from

Parallel drug selections were initiated using 0.1 nmol/L cabazitaxel, a concentration that would inhibit growth in MCF-7 cells by 50% (IC₅₀ value) with and without 2 μmol/L PSC-833. Selections continued by increasing the drug concentration in a stepwise manner up to a final concentration of 5 nmol/L cabazitaxel. Variants were grown drug-free for at least two passages before experiments.

The doxorubicin-selected human uterine sarcoma MDR variant MES-SA/Dx5 (authenticated and submitted to the ATCC as CRL-1977) was used as a positive control for transporter activity (11). In addition, two docetaxel-selected MCF-7 variants were used in this study. The MCF-7/TXT50 variant was selected with docetaxel alone, is positive for P-gp and demonstrates a typical MDR phenotype. The MCF-7/TXT50 variant was coselected with docetaxel and PSC-833, and its resistance is not due to transporters (4).

Growth inhibition assays

The in vitro activity of various anticancer drugs was tested using a modified sulforhodamine B (SRB) colorimetric assay following a 72-hour drug incubation representing approximately three cell divisions (4). Clonogenic assays were also used to assess cell survival and proliferation. In these assays, 5 to 10 x 10⁵ cells were seeded in 6-well tissue culture dishes and allowed to attach overnight. Cells were exposed to increasing concentrations of taxane (0.1 nmol/L to 1 μmol/L) for 24 hours, at which time the medium containing drug was aspirated and replaced with drug-free complete medium. Cells were incubated for an additional 14 days at 37°C and 5% CO₂ fixed in 10% (v/v) trichloroacetic acid overnight at 4°C, stained with a 0.4% (w/v) SRB solution in 1% (v/v) acetic acid, and colonies greater than 50 cells per aggregate were scored. Drug effects were calculated as a percentage relative to untreated control survival, and response versus drug concentration was calculated using the Hill equation in KaleidaGraph software (Synergy Software). Each drug concentration was tested in quadruplicate measurements per experiment, and data presented are the average of three independent experiments ± SDs.

Gene-expression profiling by quantitative PCR and NanoString Technology

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and 1 μg was reverse transcribed (RT) into cDNA using the Super Script III First-Strand Synthesis Kit with oligo(dt)₁₆ primer (both from Life Technologies), and the resulting cDNA was stored at −20°C. Expression of genes of interest was measured by quantitative PCR (qPCR; ref. 4) using a QuantStudio 12K Flex Real-Time PCR system (Life Technologies).

A highly sensitive, multiplexed measurement of gene expression was also used. NanoString Technology is based on a color-coded barcode (nCounter Reporter Probes) attached to a single target-specific probe corresponding to a gene of interest, which is hybridized directly to target molecules without the need for amplification (12). A custom-designed code set was used, and each reaction contained 250 ng of total RNA in a 5 μL aliquot. Probes were added in massive excess to target mRNA to ensure that each gene would be labeled, followed by a series of wash steps to remove unbound probes and nontarget cellular transcripts. Color codes were counted and tabulated for each target molecule using the nCounter Digital Analyzer (NanoString Technologies) at Stanford’s Functional Genomics Facility. Raw counts were normalized to internal levels of seven reference genes, and a background count was estimated using the average count of eight negative control probes in every reaction.

Western blotting

Total protein lysates were isolated from growing cells using 1× radioimmunoprecipitation assay buffer [RIPA, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS in 1× PBS buffer] with freshly added protease inhibitors (cocktail from Bio-Rad Laboratories). Total protein (10–25 μg) was separated by 4% to 20% (w/v) gradient polyacrylamide gels and transferred onto nitrocellulose membranes using the Trans-Blot Turbo transfer system (all Bio-Rad Laboratories). Membranes were blocked overnight at 4°C in 1× TBS containing 5% (w/v) nonfat milk and 1% (w/v) BSA, and then incubated with the following antibodies: anti-P-gp, BCRP, and MRP2 (Signet Laboratories); anti-MRP7 (Thermo Fisher Scientific); anti-class I, pan-α- and β-tubulin (Sigma-Aldrich); anti-class II and III β-tubulin (Covance); class IV β-tubulin (Abcam); anti-GAPDH (Santa Cruz Biotechnology); and specific antibodies for BRCA1, Bcl2, inhibitors of apoptosis (IAP), and p21 (Cell Signaling Technology). These primary antibodies were recognized by species-appropriate horseradish peroxidase–conjugated secondary antibodies, and detected using the Clarity Western ECL substrate (Bio-Rad Laboratories).

Tubulin polymerization assays

Soluble and polymerized tubulin fractions were separated by centrifugation (20,000 x g) following a 5-minute incubation in hypotonic buffer with and without drug at 37°C (13, 14). The soluble tubulin fractions were transferred to fresh microcentrifuge tubes and stored on ice, whereas fractions containing polymerized tubulin were sonicated for 10 seconds on ice before adding 4× Laemmli sample buffer (Bio-Rad Laboratories). Equal volumes of soluble and polymerized fractions were resolved on gradient polyacrylamide gels and transferred to nitrocellulose as previously described. Immunoblotting with a pan-α-tubulin antibody (clone DM1A; Sigma-Aldrich) isolated the tubulin fractions, and the percentage of tubulin polymer present in each fraction was calculated on the basis of the total tubulin (soluble and polymerized) present in each experimental condition as determined by densitometry.

Functional assays for transporter activity

Cellular drug accumulation was determined using a published method (6). Briefly, 1 x 10⁶ cells were seeded in 6-well dishes and...
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allowed to attach overnight. [3H]-docetaxel (10 nmol/L American Radiolabeled Chemicals) was allowed to accumulate for 1 hour at 37°C with and without 2 μmol/L PSC-833, aspirated, and dishes were washed once with ice-cold PBS. Cells were lysed immediately using a 2% (w/v) SDS solution, and counts were determined upon the addition of Ecolite liquid scintillation cocktail (MP Biomedical), and normalized to protein content.

These data were confirmed by determining the accumulation of rhodamine-123 and BODIPY–paclitaxel (both Life Technologies) by flow cytometry. Cells were harvested, exposed to either rhodamine-123 or BODIPY–paclitaxel for 1 hour at 37°C, drug was removed by centrifugation (200 × g) at 4°C, and cells washed once in cold PBS. To correlate drug accumulation with P-gp content, cells were stained on ice using an anti-P-gp mouse monoclonal antibody that detects an external epitope (clone UIC2; EMD Millipore), and detected by a Texas-Red goat antimouse secondary antibody (Life Technologies) using an LSR II flow cytometer (BD Biosciences). The effects of 2 μmol/L PSC-833 and other known MDR modulators were assessed in separate experimental conditions.

Transient TUBB3 and BRCAl silencing by small interfering RNA

Pools of four gene-specific siRNAs were designed using Dharmacon siDesign Center algorithm and synthesized (ON-TARGETplus reagents; GE Dharmacon). Lipid-mediated siRNA delivery into cells was accomplished with DharmaFECT 1 transfection reagent (GE Dharmacon) according to the manufacturer’s protocol. Cells were maintained in complete medium for 24 hours after transfection. Optimal concentrations of siRNAs were determined to avoid off-target effects, and the time course of gene silencing was evaluated relative to nontargeting controls (GE Dharmacon) from 24 to 96 hours after transfection by RT-qPCR and immunoblotting with specific antibodies.

Results

Cabazitaxel is more potent than paclitaxel and less cross-resistant in MDR variants

Cabazitaxel potency was measured in several taxane-sensitive cell lines using the SRB colorimetric cell proliferation assay. Cabazitaxel was 10-fold more potent than paclitaxel and comparable with docetaxel in MCF-7 breast cancer cells following a 72-hour drug incubation (Supplementary Fig. S1B). These data were confirmed in several other cell lines, including the human uterine sarcoma, MES-SA, human ovarian cancer cell lines ES-2, MES-OV, and OVCAR-3 cell lines (Supplementary Table S1).

We evaluated cabazitaxel activity in P-gp–expressing cell variants, including the doxorubicin-selected variant, MES-SA/DX5, and the docetaxel-selected MCF-7/TxT50. The expression of ABCB1 in these variants and other resistant models is shown in Fig. 1A. The MES-SA/DX5 cell line is approximately 200-fold resistant to the taxanes, paclitaxel, and docetaxel, but 15-fold resistant to cabazitaxel (Table 1). Likewise, the MCF-7/TxT50 is 60-fold resistant to paclitaxel and docetaxel, and 8.6-fold resistant to cabazitaxel. Coincubation with the P-gp inhibitor PSC-833 (2 μmol/L) completely restored sensitivity to parental levels for all taxanes tested. A non-MDR docetaxel-selected variant, MCF-7/TxTP50, was developed by coselection with docetaxel and PSC-833. These cells have elevated TUBB3 levels and altered tubulin dynamics as a mechanism of resistance, and do not have mutations in beta-1 (β-1) tubulin (class I, M40) and alpha-tubulin (K-α1). The MCF-7/TxTP50 cell line is cross-resistant to cabazitaxel (2.0-fold), comparable with the other taxanes tested, and this resistance to cabazitaxel was not affected by the MDR modulator PSC-833 (Table 1).

Development of cabazitaxel-resistant MCF-7 variants

Two cabazitaxel-resistant variants were established by long-term selection with clinically relevant concentrations of cabazitaxel in the MCF-7 breast cancer cell line. The MCF-7/CTAX was derived by cabazitaxel exposure up to 5 nmol/L, and the predominant mechanism in this variant is activation of the ABCB1 gene. We confirmed the presence of ABCB1 transcripts by RT-PCR using several sets of ABCB1-specific amplifiers (Fig. 1A). MCF-7/CTAX cells were positive for P-gp using the monoclonal antibody UIC2 by flow cytometry (Fig. 1B). Because taxanes are substrates for other ATP-binding cassette MDR transporters, we also screened for ABCG2, ABCG2, ABCG2, MRP7, and ABCG2/BCRP and found no activation of these transporters relative to parental content following cabazitaxel selection (Supplementary Fig. S2). Of interest, ABCG2 (BCRP, MXR) was activated following docetaxel selection in both of the MCF-7 variants, MCF-7/TxT50 and MCF-7/TxTP50 (Supplementary Fig. S2).

In a parallel selection, we coselected MCF-7 cells with cabazitaxel and PSC-833 and established the MCF-7/CTAX-P variant, which is approximately 9-fold resistant to paclitaxel and docetaxel, and 4-fold resistant to paclitaxel by SRB assays (Table 2). This variant is negative for P-gp expression (Supplementary Fig. S2).

The functional status of the transporter was assessed by accumulation assays using known P-gp substrates. The P-gp–positive MCF-7/CTAX cells had lower levels of [3H]-docetaxel following a 1-hour accumulation at 37°C (Fig. 1C), and these levels could be restored to parental MCF-7 levels in the presence of 2 μmol/L PSC-833 and other MDR modulators. These findings were confirmed with rhodamine-123 accumulation by flow cytometry in MCF-7/CTAX cells (Supplementary Fig. S3A), compared with P-gp–negative MCF-7/CTAX-P cells (Supplementary Fig. S3B). MCF-7/CTAX also demonstrates reduced BODIPY–paclitaxel accumulation under identical experimental conditions (Supplementary Fig. S3C), and is cross-resistant to a number of P-gp substrates, including other taxanes, vinca alkaloids, colchicine, and the anthracyclines doxorubicin and daunorubicin (Table 2). MCF-7/CTAX cells were 33-fold resistant to cabazitaxel, 52-fold resistant to paclitaxel, and 58-fold resistant to docetaxel. This resistance to taxanes was modulated in the presence of PSC-833, but 3-fold residual resistance remained to all taxanes tested, indicating that MDR was not the sole mechanism of resistance to cabazitaxel in these cells.

Taxane resistance in the MCF-7/CTAX-P variant does not appear to be transporter-mediated because it accumulates levels of [3H]-docetaxel similar to parental cells (Fig. 1C), as well as rhodamine-123 and BODIPY–paclitaxel as measured by flow cytometry (Supplementary Fig. S3B and S3D, respectively). PSC-833 did not modulate taxane resistance or the accumulation of drugs in MCF-7/CTAX-P cells. These cells are hypersensitive to the vinca alkaloids and colchicine, indicating an alteration in microtubule dynamic instability. No change in sensitivity to anthracyclines or platinum agents was detected by SRB assays.
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NOTE: SRB assays were run following 72 hours of drug incubations with and without PSC-833 (2 determinations/C6 for P-gp status in MCF-7/CTAX (B, gray shaded area) and MCF-7/CTAX-P (dashed gray line) compared with MCF-7 parental cells (solid black line) by MCF-7/TxTP50 variants were included as a negative control. UIC2 staining recognized by a Texas Red-conjugated secondary antibody was used to screen [3H]-docetaxel levels were determined following a 1-hour accumulation at 37°C addition, the docetaxel-selected variant MCF-7/TxT50 was included as another positive control for Parental MES-SA cells and doxorubicin-selected MES-SA/Dx5 variants were included as negative and positive controls for MCF-7/CTAX-P (gray shaded area) relative to the MCF-7 control (solid black line) by observe a reduction in bound fluctuation in MCF-7/CTAX-P relative to parental controls, we did TxTP50 variant, which was selected for resistance to docetaxel in the presence of the P-gp inhibitor PSC-833 aRelative resistance following coincubation with the P-gp inhibitor PSC-833 at 2 bRelative resistance following coincubation with the P-gp inhibitor PSC-833 at 2

Figure 1. The expression of ABCB1 was determined by RT-PCR following 35 cycles of amplification, with ribosomal RNA-specific amplimers used as an internal control (A). Parental MES-SA cells and doxorubicin-selected MES-SA/Dx5 variants were included as negative and positive controls for ABCB1 expression, respectively. In addition, the docetaxel-selected variant MCF-7/TxT50 was included as another positive control for ABCB1 transcripts, and the docetaxel- and PSC-833-coselected MCF-7/TxTP50 variants were included as a negative control. UIC2 staining recognized by a Texas Red-conjugated secondary antibody was used to screen for P-gp status in MCF-7/CTAX (B, gray shaded area) and MCF-7/CTAX-P (dashed gray line) compared with MCF-7 parental cells (solid black line) by flow cytometry. [3H]-docetaxel levels were determined following a 1-hour accumulation at 37°C with and without 2 μmol/L PSC-833 and normalized to protein content (C). The average of three determinations is presented ± SD. BODIPY-paclitaxel binding was assessed following a 1-hour efflux at 37°C after drug accumulation in MCF-7/CTAX-P (gray shaded area) relative to the MCF-7 control (solid black line) by flow cytometry (D). Untreated MCF-7 (dashed gray line) is included for reference.

Although we did not detect any differences in taxane accumulation in MCF-7/CTAX-P relative to parental controls, we did observe a reduction in bound fluorescent-labeled paclitaxel using flow cytometry. After accumulation in BODIPY-paclitaxel for 1 hour at 37°C, cells were allowed to efflux in drug-free complete medium for an additional hour and washed several times to insure that no unbound drug remained. MCF-7/CTAX-P cells had a 34% decrease in residual bound BODIPY-paclitaxel as measured by average fluorescence intensity compared with bound drug measured in parental MCF-7 cells (Fig. 1D). Incorporating PSC-833 in the drug treatment did not affect the degree of drug binding in this cell line.

Table 1. Cabazitaxel activity in two ABCB1 variants, the doxorubicin-selected MES-SA/Dx5 and docetaxel-selected MCF-7/TxT50, and in the non-ABCB1 MCF-7/TxTP50 variant, which was selected for resistance to docetaxel in the presence of the P-gp inhibitor PSC-833

<table>
<thead>
<tr>
<th>ABCB1 and P-gp</th>
<th>Relative resistance (modulation by PSC-833)</th>
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<tbody>
<tr>
<td></td>
<td>(+) MES-SA/Dx5</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>190 ± 20 (2.2 ± 0.76)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>210 ± 11 (1.5 ± 0.20)</td>
</tr>
<tr>
<td>Cabazitaxel</td>
<td>15 ± 1.7 (0.80 ± 0.30)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>150 ± 21 (1.5 ± 0.25)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>150 ± 26 (1.3 ± 0.20)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>170 ± 29 (1.2 ± 0.23)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>35 ± 2.5 (1.5 ± 0.20)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>32 ± 2.0 (1.3 ± 0.20)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.0 ± 0.5 (1.5 ± 0.30)</td>
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</table>

NOTE: SRB assays were run following 72 hours of drug incubations with and without PSC-833 (2 μmol/L). Data are expressed as the mean of three independent determinations ± SDs.

*The relative resistance was calculated by dividing the IC50 value of the variant by the IC50 value of the wild-type cell line.

Relative resistance following coincubation with the P-gp inhibitor PSC-833 at 2 μmol/L.
Immunoblotting with a pan-β-tubulin antibody (clone DM1A; Sigma-Aldrich) confirmed the tubulin content relative to parental controls, and this effect was dose-dependent with increased drug exposure, especially with cabazitaxel and docetaxel. We achieved greater than 90% silencing, reversing the TUBB3 content back to parental levels (Fig. 3A). Clonogenic assays demonstrated that cells were approximately 2-fold more sensitive to cabazitaxel than the parental cell line (Fig. 1B). This impaired tubulin polymerization in response to drug treatment correlated with resistance to the taxanes in cytotoxicity assays, and may be due to the reduced binding of taxanes to microtubules observed in MCF-7/CTX-P.

Expression of the class III (TUBB3) β-tubulin isotype, total α- and β-tubulin levels, and BRCA1 was evaluated by immunoblotting total cell lysates from MCF-7, the ABCB1-positive MCF-7/CTX and the coselected MCF-7/CTX-P cell lines (A). Protein loading was confirmed by screening for β-actin expression. Tubulin polymer was separated from soluble tubulin by centrifugation (20,000 × g) following a 5-minute incubation in hypotonic buffer with and without drug at 37°C, and fractions were resolved on 10% to 20% gradient polyacrylamide gels and transferred to nitrocellulose. Immunoblotting with a pan-α-tubulin antibody (clone DM1A; Sigma-Aldrich) isolated the tubulin fractions in MCF-7 parental cells and the MCF-7/CTX-P variant (B).

Table 2. Resistance phenotype in the ABCB1-positive MCF-7/CTX and in the non-MDR MCF-7/CTX-P variant, which was coselected with cabazitaxel and PSC-833

<table>
<thead>
<tr>
<th>ABCB1 and P-gp</th>
<th>Relative resistance (modulation by PSC-833)</th>
<th>MCF-7/CTX</th>
<th>MCF-7/CTX-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabazitaxel</td>
<td>(−)</td>
<td>33 ± 3.8</td>
<td>37 ± 5.8</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>(−)</td>
<td>58 ± 4.5</td>
<td>61 ± 5.0</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>(−)</td>
<td>52 ± 2.5</td>
<td>43 ± 3.0</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>(−)</td>
<td>35 ± 5.0</td>
<td>35 ± 6.0</td>
</tr>
<tr>
<td>Vincristine</td>
<td>(−)</td>
<td>37 ± 3.6</td>
<td>37 ± 4.0</td>
</tr>
<tr>
<td>Colchicine</td>
<td>(−)</td>
<td>4.3 ± 0.40</td>
<td>4.3 ± 0.40</td>
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<tr>
<td>Daunorubicin</td>
<td>(−)</td>
<td>3.2 ± 0.29</td>
<td>3.2 ± 0.29</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>(−)</td>
<td>3.0 ± 0.50</td>
<td>3.0 ± 0.50</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>(−)</td>
<td>1.3 ± 0.20</td>
<td>1.3 ± 0.20</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>(−)</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>(−)</td>
<td>0.42</td>
<td>0.30</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>(−)</td>
<td>0.29</td>
<td>0.21</td>
</tr>
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</table>

NOTE: Assays were run after 72 hours of drug incubations with and without PSC-833 (2 μmol/L). Data are expressed as the mean of three independent determinations ± SD.

aRelative resistance following coincubation with the P-gp inhibitor PSC-833 at 2 μmol/L.

bRelative resistance following coinubation with the P-gp inhibitor PSC-833 at 2 μmol/L.

Under the experimental conditions of our tubulin polymerization assay, the majority of the tubulin was present in the soluble form in our cell lines. However, we detected reduced tubulin polymer in MCF-7/CTX-P cells compared with MCF-7 cells at baseline, and the ratio of polymerized to soluble tubulin was markedly decreased (12% vs. 37%, respectively, Fig. 2B and Supplementary Fig. S4). To determine the effects of taxane treatment on tubulin polymerization, we exposed cells to either cabazitaxel or docetaxel from 1.0 nmol/L to 10 μmol/L. We observed a dose-dependent increase in levels of polymerized tubulin in response to taxane treatment in parental cells, but there was a significant difference in polymerized tubulin after cabazitaxel or docetaxel treatment at concentrations less than 1 μmol/L in MCF-7/CTX-P versus the parental cell line (Fig. 2B). This impaired tubulin polymerization in response to drug treatment correlated with resistance to the taxanes in cytotoxicity assays, and may be due to the reduced binding of taxanes to microtubules observed in MCF-7/CTX-P.

TUBB3 gene silencing sensitizes MCF-7/CTX-P cells to cabazitaxel

To test the role of TUBB3 expression on cabazitaxel activity, we transfected MCF-7/CTX-P cells with a pool of four siRNAs specific for the class III β-tubulin. We achieved greater than 90% silencing, reversing the TUBB3 content back to parental levels (Fig. 3A). Clonogenic assays demonstrated that cells were approximately 2-fold more sensitive to cabazitaxel than the control transfected with a pool of nontargeting siRNAs (Fig. 3B). In a parallel experiment, we silenced TUBB3 in parental MCF-7 cells using the same pool of siRNAs under the same transfection conditions. TUBB3 was undetectable by immunoblotting following transfection, and these cells were 1.3-fold more sensitive to both cabazitaxel and docetaxel compared with the nontargeting control (data not shown). Data from these experiments indicate that TUBB3 is at least partially responsible for the resistance to cabazitaxel in MCF-7/CTX-P cells.

Reduced BRCA1 expression in response to cabazitaxel drug selection, and resistance to cabazitaxel by BRCA1 silencing in MCF-7 parental cells

We have previously reported downregulation of BRCA1 resulting from either paclitaxel or docetaxel selection in several models of non-ABC1 taxane resistance. Reduced BRCA1 content was observed early in taxane selections, usually during the first round of drug treatment, and these alterations were associated with decreased G2–M arrest and apoptosis induced by taxane treatment (15). Selection with cabazitaxel in MCF-7 cells also resulted in BRCA1 downregulation in both MCF-7 variants, but we observed lower levels of BRCA1 in the non-ABC1 MCF-7/CTX-P (70% reduced compared with MCF-7) than in MCF-7/CTX (35% reduced, Fig. 2A). To evaluate the role of BRCA1 downregulation in cabazitaxel resistance, we silenced the gene in the parental MCF-7 cell line and measured effects on taxane cytotoxicity. Treatment with a BRCA1-specific pool of four siRNAs achieved greater than 90% silencing compared with parental and a nontargeting control by immunoblotting (Fig. 3C), and BRCA1-silenced MCF-7 cells were approximately 4-fold resistant to cabazitaxel (Fig. 3D) relative to the nontargeting control. These
observations were confirmed following treatment with docetaxel after BRCA1 silencing (data not shown). Thus, BRCA1 expression is directly implicated in cellular responses to taxanes in MCF-7 cells.

Altered epithelial–mesenchymal and apoptosis regulating genes

Gene-expression profiling using the NanoString Technologies platform identified alterations in the expression of epithelial–mesenchymal transition (EMT) markers in both cabazitaxel-selected variants, with elevated levels of the mesenchymal marker, Vimentin (VIM), and decreased expression of the epithelial cell–cell adhesion glycoprotein E-cadherin (CDH1) compared with parental cells (Fig. 4). Therefore, cabazitaxel selection induces EMT, implicating the mesenchymal phenotype in resistance to cabazitaxel.

Although altered expression of apoptotic regulators has been associated with taxane resistance in other published models (16, 17), gene-expression profiling using the NanoString platform indicated that there were no significant changes in the apoptotic promoters BAX and BAD, and we observed reduced expression of the antiapoptotic regulators BCL2, MCL1, and several IAPs, including cIAP-1/BIRC2, cIAP-2/BIRC3, XIAP/BIRC4, Survivin/BIRC5, and Livin/BIRC7 in the cabazitaxel-selected variants compared with MCF-7 parental cells (Fig. 4). These findings were confirmed by immunoblotting with specific antibodies, and we did not detect a difference in Bcl-XL protein levels in either cabazitaxel variant compared with MCF-7 levels (Supplementary Fig. S5). Furthermore, several DNA repair genes were downregulated in both cabazitaxel variants such as the DNA excision repair ERCC1 and Fanconi anemia group F (FANCF) genes, and we observed alterations in genes associated with detoxification, including reduced glutathione S-transferase P1 (GSTP1) and elevated glutathione peroxidase 3 (GPX3) genes (Fig. 4).

Discussion

Cabazitaxel was selected for clinical development due to its activity in tumor models that were demonstrated to be poorly sensitive or insensitive to docetaxel treatment, including lung, and...
Resistance to Cabazitaxel

Alterations in microtubule composition and dynamics have been reported in taxane variants (1, 21–23). Previous data from our laboratory indicated that an increased expression of the class IVa (TUBB4) β-tubulin isotype was associated with non-ABCB1 taxane resistance in a human leukemia variant coselected with paclitaxel and PSC-833 (6). No other changes in β-tubulin isotype content, including mutations in the putative taxane-binding region, were observed in this cell line that was exclusively resistant to taxanes, and total α- and β-tubulin levels remained unchanged. In addition, the majority of our taxane-resistant non-ABCB1 breast and ovarian cancer models have elevated class III (TUBB3) content following selection with either docetaxel or paclitaxel in the presence of PSC-833, and we demonstrated that the MCF-7/TxTP50 variant is also cross-resistant to cabazitaxel.

Others have reported that elevated levels of class III β-tubulin conferred resistance to taxanes (21, 24–26). Hari and colleagues (24) generated clones of the Chinese hamster ovary cell line that had tetracycline-controlled expression of the class III β-tubulin isotype. Stable clones with high levels of class III had significantly lower proliferation rates compared with wild-type cells and with cells grown in the presence of tetracycline. These high levels of class III β-tubulin were found to be cytotoxic, diminishing microtubule assembly in transfected cells, and were found to confer modest resistance (1.5- to 2-fold) to paclitaxel over controls. As with our results in the MCF-7 breast cancer cell line, several others have observed slight sensitization to taxanes following transient TUBB3 silencing using specific siRNAs or antisense oligonucleotides (27–29). Although several published reports have questioned the importance of β-tubulin isotype expression as a significant mechanism of resistance to paclitaxel (30–32), TUBB3 content appears to predict paclitaxel response in breast, non–small cell lung and other cancers, with elevated TUBB3 levels correlated with poor prognosis and clinical outcome in patients treated with taxanes (28, 33–37). Our study demonstrated that transient TUBB3 silencing resulted in modest sensitization to cabazitaxel in the MCF-7/TxTP50 cell line.

Furthermore, expression of cell-cycle regulators has been implicated in models of taxane resistance. In experiments testing the functional significance of these alterations, transfection of active, wild-type BRCA1 in BRCA1-negative HCC1937 human breast cancer cells sensitized cells to taxanes (38), whereas both transient and stable silencing of BRCA1 resulted in taxane resistance in a number of cell models, including breast, ovarian, and lung cancer cell lines (38–43). A recent study found that BRCA1 regulates microtubule dynamics, with reduced taxane binding impairing drug-induced microtubule stabilization, resulting in resistance to taxanes in A549 lung cancer cells transfected with a BRCA1-specific shRNA relative to parental and empty vector controls (43). Kurebayashi and colleagues (44) found that loss of BRCA1 expression may predict a shorter progression-free survival time in breast cancer patients treated with taxanes. Reduced BRCA1 expression was also associated with cabazitaxel resistance in our MCF-7 variants, and transient silencing of BRCA1 by siRNA transfection in MCF-7 parental cells resulted in 4-fold resistance to cabazitaxel. The altered taxane binding observed in the MCF-7/CTAX-P variant may result from its reduced tubulin polymer content compared with parental MCF-7 cells (14, 45).

The acquisition of mesenchymal properties has been associated with chemoresistance, including upregulation of TUBB3 and resistance to taxanes (46–48). EMT is characterized by the loss of the epithelial and the gain of the mesenchymal markers, and we...
observed these alterations in both MCF-7 variants following cabazitaxel selection, indicating that resistance to cabazitaxel is associated with a more aggressive and invasive phenotype. The functional significance of these changes in marker expression is being studied in the laboratory. The blockade of EMT may modulate taxane resistance in these cell models of cabazitaxel resistance. We found substantial cross-resistance between cabazitaxel and the first-generation taxanes, paclitaxel and docetaxel, despite the higher potency of cabazitaxel and its lower degree of resistance in P-gp–positive resistant variants. Thus, in lieu of data from appropriate clinical trials, our data should not be interpreted to support the use of cabazitaxel in docetaxel-refractory breast cancers. This study has identified several potential biomarkers for predicting cabazitaxel effectiveness in the clinical setting, including reduced BRCA1 and elevated class III microtubule-stability factors.

Disclosure of Potential Conflicts of Interest

G. E. Duran, Y. C. Wang, E. B. Francisco, J. C. Rose, F. J. Martinez, J. Coller, and B. I. Sikic report receiving a commercial research grant from Sanofi Oncology. P. Vrignaud is an employee and stock holder at Sanofi. No potential conflicts of interest were disclosed by the author D. Brassard.

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George E. Duran, Yan C. Wang, E. Brian Francisco, et al.


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