Expression and Silencing of the Microtubule-Associated Protein Tau in Breast Cancer Cells

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Abstract

The microtubule-associated protein Tau has been reported to be a predictive factor for clinical response to taxanes in metastatic breast cancer. We generated a panel of eight taxane-resistant variants from four human breast cancer cell lines (MCF-7, T-47D, MDA-MB-231, and BT-549). Four variants had higher levels of Tau compared with their T-47D and MDA-MB-231 parental cells. Using isoform-specific primers, we found that Tau 0N, 1N, 2N, 3R, and 4R isoforms are overexpressed in the resistant variants, as is Tau exon 6 but not exons 4A or 8. To determine whether Tau overexpression produces resistance to taxanes, we derived three independent T-47D clones stably overexpressing Tau 3R and 4R isoforms. Tau overexpression did not result in taxane resistance compared with parental cells transfected with vector alone. We then knocked down Tau expression in three cell lines that expressed Tau constitutively (MCF-7 and ZR-75-1 breast cancer cells, and OVCAR-3 ovarian cancer cells). Lentivirus-mediated silencing of Tau expression in MCF-7 and OVCAR-3 cells did not result in increased taxane sensitivity compared with luciferase short hairpin RNA–infected cells and uninfected parental cells. Transient silencing using Tau-specific small interfering RNAs also did not alter taxane sensitivity relative to nontargeting controls in both MCF-7 and ZR-75-1 cells. These results show that neither overexpression nor depletion of Tau modulates cellular sensitivity to taxanes. Although Tau overexpression has been reported to be a predictive marker of taxane resistance, it is not likely to be a direct mechanism of taxane resistance in breast cancer.

Introduction

The taxanes paclitaxel and docetaxel are an important class of microtubule-stabilizing drugs in the treatment of solid tumors, including breast cancer (1). In breast cancer, taxanes have shown clinical benefit both as adjuvant chemotherapy for localized disease (2, 3) and in the treatment of metastatic disease (4). However, as is the case for almost all anticancer agents, inherent or acquired taxane resistance greatly limits their clinical utility (5–7).

Resistance to taxanes can involve multiple diverse mechanisms, including increased drug efflux, altered microtubule dynamics, or impaired cell death signaling. Drug efflux mediated by the overexpression of the multidrug transporter P-glycoprotein (P-gp), the gene product of MDR1 (ABCB1), is an important resistance mechanism for many chemotherapeutic agents, including taxanes (8). However, the majority of clinically resistant breast cancers are P-gp negative (9), and therefore manifest other mechanisms of resistance to taxanes.

Several lines of evidence suggest that the microtubule-associated protein (MAP) Tau may play a role in modulating response to taxanes. (a) Tau was one of the differentially expressed genes associated with response to preoperative paclitaxel chemotherapy in breast cancer patients (10, 11). (b) Tau expression across 23 cancer cell lines correlated with IC50 values for paclitaxel (12). (c) Downregulation of Tau expression in ZR-75-1 and MCF-7 breast cancer cell lines was reported to increase sensitivity to paclitaxel (10). (d) Reduced Tau expression in gastric cancer was associated with a favorable response to paclitaxel (13).

Tau is a protein enriched in axons of mature and growing neurons whose primary function is to regulate microtubule dynamics (14). Tau is also found in the distal ends of growing neurons (15, 16), in oligodendrocytes (17, 18), and in muscle (19). It is encoded by a 100-kb single gene on chromosome 17q21 (20), consisting of 16 exons, of which exons 2, 3, 4A, 6, 8, 10, 13, and 14 are alternatively spliced (19, 21–28). Alternative splicing of three major exons (2, 3, and 10) results in the expression of six well-characterized Tau isoforms in the human brain (Fig. 1A). Although Tau has been identified as a potential marker of paclitaxel response in breast cancer (10, 11), its functional significance and isoform characterization have not been fully investigated.

The aim of this study was to evaluate the functional role of Tau in modulating taxane response and to characterize...
the expression of Tau isoforms in taxane-resistant breast cancer cells. Our results indicate that although Tau isoforms are overexpressed in some taxane-resistant breast cancer cells, neither downregulation nor overexpression of Tau alters cellular sensitivity to taxanes.

Materials and Methods

Materials
Paclitaxel was obtained from the drug repository of the National Cancer Institute (Bethesda, MD), docetaxel was from Sanofi-Aventis, and PSC-833 was from Novartis (Fig. 2). All other chemicals were purchased from Sigma-Aldrich.

Cell lines and cultures
Human cancer cell lines MCF-7, BT-549, MDA-MB-231, T-47D, ZR-75-1, and OVCAR-3 were purchased from and characterized by the American Type Culture Collection. All cells were cultured in McCoy’s 5A medium with t-glutamine, supplemented with 10% (v/v) FCS, penicillin, and streptomycin (all from Invitrogen). Cells were maintained at 37°C in an atmosphere containing 5% CO₂.

T-47D cells transfected with plasmids encoding Tau 3R and 4R isoforms were selected and maintained in complete McCoy’s 5A medium containing 500 μg/mL G418 (Invitrogen).

Total RNA isolation and cDNA synthesis
Total RNA was extracted using RNeasy Mini kit (Qiagen), resuspended in RNase-free water, and stored at −80°C until analysis. Total RNA (1–5 μg) was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis kit with the oligo(dT)₂₀ primer (both from Invitrogen). The final cDNA product was stored at −20°C until further analysis.
Real-time PCR
Quantitative real-time PCR was carried out in duplicate or triplicate per experiment, with each experiment reproduced multiple times using SYBR Green PCR Master Mix on an ABI 7900HT real-time PCR instrument (Applied Biosystems). A PCR mixture of 20 μL contained 10 μL of 2× SYBR Green PCR Master Mix, 3.5 μL of 0.75 μmol/L gene-specific forward primer, 3.5 μL of 0.75 μmol/L gene-specific reverse primer, and 3 μL cDNA. The amplification program consisted of 1 cycle at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, annealing temperature at 60°C for 30 seconds, and extension temperature at 72°C for 15 seconds. Standard curves for each of the six known Tau isoforms and for HPRT1 target transcript. Primers for amplification of Tau exons were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA), summarized as follows: 0N, 5′-GCTGGCCTGAAACGCTGAAG-3′ (forward) and 5′-GTCTTCTTCGAGTGCGCATCT-3′ (reverse); 1N, 5′-CACACAGGCGGCTGGAAGAAGA-3′ (forward) and 5′-GTGACCCAGCTGCTGGTAGT-3′ (reverse); 2N, 5′-ACTCCCAACAGCGAGATGT-3′ (forward) and 5′-GTGACCCAGCTGCTGGTAGT-3′ (reverse); 3R, 5′-AGGCCGGAAGTCGCGAAATTTAA-3′ (forward); 5′-GCCACCCCTCCTGGTTTATGATG-3′ (forward) and 4R, 5′-CGGGAAGTGCGAGATTTAAA-3′ (forward) and 5′-TATTGGCACACTGCGCCCT-3′ (reverse).

End-point PCR
Primers for amplification of Tau exons were designed using the Primer3 software. Primers for the TFRC housekeeping gene were purchased from http://RealTimePrimers.com. All PCRs were done in a final volume of 10 μL, consisting of 1 μL of 10× PCR buffer, 1 μL of RediLoad Dye (Invitrogen), 2.23 μL of each Tau isoform-specific primer (300 nmol/L final concentration), 1 μL of endogenous control TFRC primer mix, 0.2 μL of deoxynucleotide triphosphates, 0.3 μL of MgCl2, 2 μL of 1:5 diluted cDNA, and 0.04 μL Platinum Taq. The amplification program consisted of 1 cycle at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, annealing temperature at 60°C for 30 seconds, and extension temperature at 72°C for 15 seconds. The PCRs were analyzed by gel electrophoresis, and the products were visualized using the SYBR Safe DNA gel stain (Invitrogen). The primer sequences and target regions are summarized as follows: 0N + exon 6, 5′-GCTGGCCTGAAACGCTGAAG-3′ (forward) and 5′-GTCTTCTTCGAGTGCGCATCT-3′ (reverse); 1N + exon 6, 5′-CACACAGGCGGCTGGAAGAAGA-3′ (forward) and 5′-GTGACCCAGCTGCTGGTAGT-3′ (reverse); 2N + exon 6, 5′-ACTCCCAACAGCGAGATGT-3′ (forward) and 5′-GTGACCCAGCTGCTGGTAGT-3′ (reverse); and exons 6 and 8, 5′-CCCAGTGCTGAAACGCTGAAG-3′ (forward) and 5′-GTCTTCTTCGAGTGCGCATCT-3′ (reverse).

Western blotting
Total protein lysates were isolated from growing cells using 1× radioimmunoprecipitation assay buffer [1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS in 1× PBS buffer] with freshly added protease inhibitors (phenylmethylsulfonyl fluoride and aprotinin). Total protein (10–25 μg) was separated by 4% to 12% Nu-PAGE gels and transferred onto nitrocellulose membranes using the iBlot transfer system (all Invitrogen). Membranes were blocked overnight at 4°C in 1× TBS containing 5% (w/v) nonfat milk and 1% (w/v) bovine serum albumin and then incubated for 2 hours at room temperature with the following antibodies: Tau T-1308-1 (rPeptide); P-gp (Signet Laboratories); MAP4 (BD Transduction Laboratories); statmin-1, class IV tubulin (Abcam); class II and III tubulin (Covance); and class I tubulin, pan α- and β-tubulin (Sigma-Aldrich). Primary antibodies were recognized by

Figure 2. Chemical structures of docetaxel and paclitaxel (A) and PSC-833 (B).
appropriate horseradish peroxidase–conjugated secondary antibodies (GE Healthcare Life Sciences) and visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Sciences). Tau protein ladder T-1007 was purchased from rPeptide.

Cell proliferation assays

The surviving fraction of cells exposed to taxanes was determined using a modified clonogenic assay (29, 30). Briefly, 6,500 cells were seeded in six-well tissue culture plates (BD Falcon) and allowed to attach overnight. Cells were exposed to increasing concentrations of taxane (either paclitaxel or docetaxel from 0.1 nmol/L to 1 μmol/L) for 24 hours, at which time the medium was aspirated and replaced with drug-free complete medium. Cells were incubated for 14 days at 37°C and 5% CO2, the surviving colonies were stained with 0.4% (w/v) sulforhodamine B (SRB) in 1% (v/v) acetic acid, and colonies (>50 cells) were counted and expressed as a percentage of an untreated control. Alternatively, a short-term SRB colorimetric cell proliferation assay was used to determine cell survival following drug exposure. In this assay, 8,000 cells were seeded in 96-well tissue culture plates (BD Falcon) and allowed to attach overnight. Drugs at relevant concentrations were added, and the plates were incubated for 72 hours, or approximately three cell divisions. Total proteins were fixed in 10% (w/v) trichloroacetic acid overnight and stained with SRB for 1 hour, and plates were washed thoroughly with 1% (v/v) acetic acid. Protein-bound dye was solubilized in a 10 mmol/L Tris base (pH 10) and plates were read in a multiwell spectrophotometer at 570 nm (Molecular Devices).

Stable transfection of Tau 3R and 4R isofoms

T-47D cells were seeded in 12-well tissue culture plates (BD Falcon) and allowed to attach overnight to achieve 90% confluency at the time of transfection. Purified plasmid DNA (1.6 μg), encoding either vector alone, EGF–Tau 3R, or EGF–Tau 4R isofoms, was transfected into cells using Lipofectamine 2000 (Invitrogen) per the manufacturer’s protocol. Cells were selected 48 hours after transfection in medium containing 500 μg/mL G418, which was replaced every 2 days. Three independent T-47D clones for each condition (empty vector, Tau 3R, and Tau 4R), which survived the G418 selection, were grown and used for subsequent experiments. The plasmids were a kind gift of Dr. Kenneth Kosik (University of California, Santa Barbara, CA).

Short hairpin RNA lentivirus–mediated knockdown of Tau and MAP4

Tau and MAP4 gene sequences were entered into the MIT/Harvard Broad Institute RNAi Consortium shRNA Library Web site (http://www.broad.mit.edu), and the following forward and reverse sequences were synthesized (Integrated DNA Technologies, Inc.): Tau, 5′-CCGGCCAGGCTTAAATACGTCTGTTTAGCTAGTAAACCATGATCTTAGGCTGTGGGTGTAACCCATGATCTTAGGCTTATGTTATCGAGTAAACCCTAGATCTTATCCGGTG3′ (reverse); MAP4, 5′-CCGGCCAGGCTTAAATACGTCTGTTTAGCTAGTAAACCATGATCTTAGGCTGTGGGTGTAACCCATGATCTTAGGCTTATGTTATCGAGTAAACCCTAGATCTTATCCGGTG3′ (reverse).

The oligonucleotides were resuspended per the manufacturer’s instructions and annealed to form a hairpin (referred to as shTau1.1 and shMAP4 4.1) under the following conditions: 94°C for 5 minutes, 70°C for 5 minutes, and drop of 0.1°C per second to 4°C. The annealed hairpins (designed with AgeI and EcoRI-compatible ends) were ligated into an AgeI/EcoRI-digested pLKO.1 vector using T4 ligase protocol per the manufacturer’s protocol (Invitrogen). The ligation products were transformed into DH5α–competent cells, and plasmids were isolated from several single colonies and sequenced. Vectors with confirmed Tau1.1 and MAP4 4.1 hairpin sequence, and pLKO.1 vector containing a short hairpin RNA (shRNA) targeting the firefly luciferase gene as a negative control were used for subsequent lentivirus production. Recombinant lentivirus was produced by co-transfecting 293 cells with the pLKO.1/shRNA-luciferase, pLKO.1/shRNA-Tau1.1, or pLKO.1/shRNA-MAP4 4.1 plasmid, along with pMDZG and D8.2 packaging plasmids, using a calcium chloride transfection method. Medium containing infectious lentiviruses was collected and filtered at 48 and 72 hours after transfection. MCF-7 or OVCAR-3 cells (1,000–1,500) were plated in 10-cm² tissue culture dishes (BD Falcon) and allowed to attach overnight. The medium was removed and replaced with 10 μL of lentivirus-containing medium plus 10 μL of Polybrene to allow infection of the cells. Forty-eight hours after infection, the medium was changed to puromycin selection medium (5 μg/mL). The infected cells were maintained in medium containing puromycin thereafter.

Transient Tau silencing by small interfering RNA in MCF-7 and ZR-75-1

Five independent Tau-specific small interfering RNAs (siRNA) were designed and synthesized (Qiagen) targeting the following sequences: MAPT_1, 5′-ccgggcaggtgagataaat-taa-3′; MAPT_4, 5′-taggaaactcatataaa-3′; MAPT_5, 5′-ccggccaggttagtgaatgt-3′; MAPT_7, 5′-ccgggagaacgg-ccaaagcctaa-3′; and MAPT_9, 5′-ccggaactaggctgacagaaa-3′. Two siRNAs (5′-aatactacacacagcag-3′ and 5′-aactggcagtgtgagcagaaa-3′) were previously reported by Rouzier et al. (10) and synthesized by Qiagen. In addition, Dharmacon’s siDesign Center (Thermo Scientific) was used to identify one additional siRNA targeting 5′-gaggaaggtgtgagtaaa-3′ of the open reading frame (regions 321–1,379). Qiagen’s AllStars negative siRNA and Dharmacon’s nontargeting siRNA were used as controls for silencing effects. Lipid-mediated siRNA delivery into cells was accomplished using Invitrogen’s RNAiMax transfection reagent. Optimal transfection conditions were determined for MCF-7 and ZR-75-1 cells, which included (a) the cell
density on the day of transfection, (b) the concentration of siRNA used, (c) the amount of RNAiMax reagent used per condition, and (d) the time course of gene silencing up to 96 hours after the introduction of siRNA.

Results

Development of taxane-resistant breast cancer variants

Four breast cancer cell lines (MCF-7, BT-549, MDA-MB-231, and T-47D) were chosen for the selection of taxane-resistant variants based on their constitutive taxane sensitivity and lack of P-gp expression. Stable taxane-resistant variants were selected by stepwise exposure to increasing doses of docetaxel over several months, with and without the MDRI inhibitor PSC-833 at 2 μmol/L. The variants are designated according to the name of the parental cell line, the taxane used for selection (TxT for docetaxel), the initial P if the cell line was coselected with PSC-833, and the concentration of taxane (e.g., MCF-7/TxTP50 is the MCF-7 variant grown in 50 nmol/L docetaxel plus

Figure 3. Development of taxane-resistant breast cancer variants. A, relative resistance of four breast cancer variants coselected by stepwise exposure to docetaxel, with and without the P-gp inhibitor PSC-833 (PSC). The highest docetaxel concentration used in the stepwise selections was 50 nmol/L. Hence, the BT-549 variant selected with docetaxel at 50 nmol/L drug concentration and PSC-833 at 2 μmol/L is designated as "BT-549/TxTP50." The resistance levels are expressed as the ratios of the IC50s of the variants to the parental cell lines, respectively. These IC50 values were determined by a 72-h SRB assay. B, P-gp expression in parental and taxane-resistant variants. The cells were selected with docetaxel, with and without PSC-833 (designated as "P"). Protein extracts were prepared from the parental and taxane-resistant cell lines, resolved by PAGE, and transferred to nitrocellulose membranes. The membranes were probed with an antibody against P-gp (C219), and an anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a loading control. All of the docetaxel-alone selections resulted in the activation of the MDRI gene, and these variants were positive for P-gp expression. All of the variants coselected with PSC-833 were P-gp negative, and drug accumulation assays determined that the taxane resistance observed was nontransporter mediated (data not shown). The doxorubicin-selected human uterine sarcoma MES-SA/Dx5 variant was used as a positive control for P-gp expression.
PSC-833). The relative taxane resistance of the four variants selected with PSC-833 was calculated by dividing the IC₅₀ of the variant by the IC₅₀ of the parental cell line (Fig. 3A). The levels of resistance to taxanes ranged from 8- to 30-fold in the PSC-833 coselected variants compared with parental cells. The four variants coselected with docetaxel and PSC-833 do not express P-gp protein as assessed by Western blotting (Fig. 3B), are negative for MDR1 transcripts by real-time PCR, and are not sensitized to taxanes by PSC-833 (data not shown).

All four resistant variants selected with docetaxel alone expressed high levels of P-gp (Fig. 3B), and the taxane resistance in these variants was completely reversed by PSC-833. Substantially higher levels of resistance to taxanes were noted in the P-gp–expressing variants selected with a docetaxel alone, ranging from 110- to 1,000-fold (data not shown).

[³H]Docetaxel intracellular accumulation was similar in the resistant variants and parental controls, indicating that the resistance observed in the variants is not related to a taxane transporter (data not shown).

### Expression of Tau protein in taxane-resistant variants

To evaluate Tau protein expression in the four parental lines and eight taxane-resistant variants, whole-cell lysates were analyzed by Western blotting (Fig. 1B). We used an antibody that recognizes all Tau isoforms and confirmed the identity of the isoforms (Fig. 1B, lane 13) by using a commercially available Tau protein ladder composed of six known Tau isoforms described in Fig. 1A. The results show that MCF-7 cells express Tau isoforms at similar levels in both the parental and taxane-resistant variants, and BT-549 cells do not express Tau in either parental or taxane-resistant variants. T-47D and MDA-MB-231 cells show elevated levels of all Tau isoforms in the taxane-resistant variants compared with parental cell lines. The overexpression of Tau in MDA-MB-231/TxTP50 and T-47D/TxTP50 variants suggests that Tau levels may be associated with taxane resistance in breast cancer.
Expression of Tau mRNA isoforms in taxane-resistant variants

Although several studies have identified Tau as a potential marker of taxane response, expression of specific Tau isoforms in breast cancer has not yet been investigated. To evaluate the levels of each Tau isoform at the mRNA level using quantitative real-time PCR assay, we designed primers that would selectively detect the following Tau isoforms: 0N Tau (no exons 2 and 3), 1N Tau (exon 2 but no exon 3), 2N Tau (both exons 2 and 3), 3R Tau (no exon 10), and 4R Tau (exon 10). To validate the isoform specificity of the primers, we used plasmids encoding each of the Tau isoforms as templates. Figure 1D shows that each of the primer pairs amplified only the targeted isoforms without significant cross-reactivity. Using the validated primers, we then measured the levels of each Tau isoform in the parental and taxane-resistant variants by quantitative real-time PCR. The standard curves for each Tau isoform were generated using serially diluted plasmids encoding a specific isoform. The amount of each isoform expression was calculated from the standard curve and normalized to the expression of HPRT1. The normalized quantity of each isoform in taxane-resistant variants was then divided by the quantity measured in parental cells to calculate fold difference. A value of 1 indicates no difference in mRNA expression, a value of >1 indicates an increase in expression in taxane-resistant variants, and no value indicates that expression was not detectable in either the parental cells or the variants. Real-time PCR assays were conducted in duplicate or triplicate for each sample on different cDNA preparations. Figure 1C shows that the expression levels of all Tau isoforms in T-47D and MDA-MB-231 taxane-resistant variants are much higher compared with parental cells, consistent with Western blot results in Fig. 1B.

Expression of Tau exons 4A, 6, and 8 in taxane-resistant variants

The Tau primary transcript contains 16 exons, with exons 4A, 6, and 8 absent in the human brain mRNA (31). Exons 4A and 6 have been found in peripheral tissues, whereas exon 8 has never been described in human tissues (14, 31). To test whether our breast cancer variants express exons 4A, 6, and 8, we did a PCR with a set of primers designed to span exons 4 and 5, exons 5 and 9, and exons 7 and 9. As a control, we coamplified the TFRC housekeeping gene in each of the reactions. We showed that exons 4A and 8 are not expressed in breast cancer cells (data not shown); however, PCR amplification spanning exons 5 and 9 (Fig. 4A) yielded products of 420 bp (Tau transcripts lacking exons 6 and 8) and 618 bp (Tau transcripts containing exon 6 but lacking exon 8). We further evaluated whether exon 6 is expressed in 0N, 1N, and 2N Tau isoforms by designing forward primers specific to 0N, 1N, and 2N and reverse primers specific to exon 7. Figure 4B to D shows that exon 6 is indeed expressed in all three isoforms.

Figure 5. Survival assay of T-47D cells overexpressing Tau after docetaxel exposure. A, SRB assays were used to determine cell survival of T-47D cells stably transfected with Tau 3R (three clones), Tau 4R (three clones), and vector alone (three clones) after drug exposure as described in Materials and Methods. Briefly, 8,000 cells were seeded in 96-well tissue culture plates and allowed to attach overnight followed by addition of drug at increasing concentrations for 72 h. Total proteins were fixed and stained with SRB, and plates were washed thoroughly and read in a multiwell spectrophotometer at 570 nm. B, each clone was assessed for protein expression of Tau, β-tubulin, and α-tubulin by Western blot analysis.
Figure 6. Sensitivity assays in Tau-silenced cells following taxane exposure. Clonogenic survival assays after exposure to paclitaxel were done in MCF-7 cells infected with Tau, luciferase, or M8AP4 shRNA (A), and OVCAR-3 cells infected with Tau or luciferase shRNA (B). Cells were seeded in six-well tissue culture plates, allowed to attach overnight, exposed to various concentrations of paclitaxel for 24 h in the presence of 2 μmol/L PSC-833, and incubated in drug-free medium for 14 d. The surviving cells were stained with SRB and colonies (>50 cells) were counted and expressed as a percentage of an untreated control. A and B, inset, Tau knockdown was confirmed by Western blot analysis. Results were confirmed using an SRB colorimetric assay following a 72-h taxane incubation. C, protein extracts from cells knocked down in Tau and MAP4 were probed for MAPs and tubulin isoforms. Actin was used as a loading control. D, treatment with a Tau-specific siRNA from Dharmacon resulted in >90% silencing in ZR-75-1 breast cancer cells. Docetaxel sensitivity was not altered in response to Tau knockdown (33 nmol/L siRNA) relative to wild-type and nontargeting controls as determined by an SRB assay. Similar results were obtained in the MCF-7 cell line under identical conditions (data not shown).
Taxane sensitivity of cells overexpressing Tau

To determine whether Tau overexpression modulates response to taxanes, we derived three independent T-47D clones stably overexpressing Tau 3R isoforms and three clones overexpressing Tau 4R isoforms. Figure 5A shows that Tau overexpression did not result in altered sensitivity to docetaxel compared with the T-47D cells transfected with empty vector alone. Additional experiments confirmed this finding in cells exposed to another taxane, paclitaxel, under identical conditions (data not shown). Furthermore, Tau overexpression did not alter the sensitivity to tubulin-depolymerizing agents such as the Vinca alkaloids, vinblastine and vincristine, and colchicine (data not shown). Tau overexpression was shown in the transfected cells by Western blotting (Fig. 5B). We did unpaired t testing of the vector-alone clones and the 3R and 4R expression clones in Fig. 5. There was no significant difference between vector and 4R clones (P = 0.2), and there was increased rather than decreased taxane sensitivity in the 3R clones (P = 0.01).

We hypothesized that perhaps the absence of observed taxane resistance may be due to upregulation of tubulin, which would allow the formation of more microtubules, and thus make more binding sites available for the drug. However, the levels of tubulin proteins were not changed between the parental and stably transfected cells (data not shown).

Taxane sensitivity of cells depleted in Tau

To further investigate whether Tau plays a functional role in modulating response to taxanes, we knocked down Tau expression in MCF-7 parental breast cancer cells and assessed survival after exposure to taxanes. We chose MCF-7 due to the high Tau expression in parental cells and applied the lentivirus-mediated shRNA approach for Tau silencing. The Tau-targeting shRNA (shTau1.1) or control luciferase shRNA (shLuc37) was cloned into a pLKO.1 vector, and lentivirus-containing medium preparation, infection, and selection process were done as described in Materials and Methods. Following selection, the infected MCF-7 cells were exposed to escalating doses of paclitaxel for 24 hours and cellular survival was assessed using clonogenic assays. Colonies (>50 cells) were counted and expressed as a percentage of an untreated control. Figure 6A shows that Tau knockdown did not result in increased sensitivity to paclitaxel compared with the luciferase shRNA–infected cells and uninfected parental cells. To confirm that Tau was indeed silenced in the infected cells, its expression was assessed by a Western blot (Fig. 6A, inset). SRB cytotoxicity assays following 72-hour drug incubations confirmed these clonogenic assay results.

Tau belongs to the same family of proteins as MAP4; therefore, we sought to determine whether silencing of MAP4 would result in altered taxane sensitivity. Lentivirus-mediated downregulation of MAP4 in MCF-7 cells also did not alter taxane sensitivity (Fig. 6A). We hypothesized that perhaps downregulation of Tau is compensated by changes in expression of other MAPs or tubulin proteins. We examined the expression of MAP4, stathmin-1, and tubulin isoforms by Western blot and did not show any significant changes in expression (Fig. 6C). To validate the finding that silencing of Tau does not sensitize cells to taxanes, we also silenced Tau in the OVCAR-3 ovarian cancer cell line (Fig. 6B). The results again showed that downregulation of Tau did not result in increased sensitivity to taxanes compared with luciferase shRNA–infected cells and parental cells.

To further test the effects of silencing Tau on taxane sensitivity, we also used a transient siRNA approach in MCF-7 and ZR-75-1 cells. We achieved ~90% Tau silencing in MCF-7 cells relative to untreated and non-targeting controls using both a fast-forward protocol (introducing the siRNA-lipid complexes immediately after plating cells) and a traditional approach where complexes are added once cells have attached. This effect was maintained for 96 hours as determined by immunoblotting, and total levels of α- and β-tubulin levels were not altered in Tau-silenced cells (data not shown). The Dharmacon–designed Tau–specific siRNA was very effective in the ZR-75-1 cell line, resulting in >90% silencing (Fig. 6D). Transient silencing did not alter taxane sensitivity in either cell line. Sensitivity to docetaxel as determined by SRB assays was not altered in ZR-75-1 cells with silenced Tau compared with untransfected and nontargeting controls (Fig. 6D). This lack of effect was substantiated using clonogenic assays following exposure to either paclitaxel or docetaxel (data not shown).

Discussion

Based on the role of Tau in microtubule stabilization, it has been hypothesized that Tau competes with taxanes for microtubule-binding sites. In this model, elevated Tau expression leads to decreased taxane binding to microtubules and, in turn, decreased taxane efficacy and suboptimal clinical response to taxane-based chemotherapy (10). However, although Tau-mediated modulation of taxane response was an intriguing hypothesis, it remained unclear whether Tau overexpression represented a true mechanism of taxane resistance or was a marker of another biological phenomenon. In addition, the role of particular Tau isoforms was poorly understood. In this study, we sought to address these questions by studying the functional role of Tau in modulating taxane response by both gene silencing and overexpression, and characterizing the expression of the various Tau isoforms in a panel of taxane-resistant breast cancer cells.

Tau is encoded by a single-copy gene, which produces three transcripts of 2, 6, and 9 kb. These transcripts are...
differentially expressed and localized depending on the cell type and stage of maturation, and produce multiple alternatively spliced isoforms. Taking into account all of the splice sites, Tau can hypothetically produce at least 30 variants by splicing alone, excluding further post-translational modifications of each isoform (19). The probe sets used in gene expression studies that originally identified Tau as a potential modulator of taxane response targeted Tau domains shared by all isoforms. We sought to characterize the expression of each of the six known major Tau splice variants in our taxane-resistant breast cancer variants (Fig. 1). Our results show that T-47D and MDA-MB-231 taxane-resistant variants express higher levels of all Tau isoforms compared with parental cells both at the protein level (Fig. 1B) and at the mRNA level (Fig. 1C).

To further characterize the expression of Tau isoforms, we sought to determine whether the breast cancer variants express exons 4A, 6, and 8, which are typically absent in the human brain. Interestingly, we showed that exon 6, but not exons 4A and 8, is expressed in 0N, 1N, and 2N Tau isoforms in breast cancer cells (Fig. 4). Exon 6 is an alternatively spliced cassette whose expression profile differs from that of other regulated exons, implying the existence of distinct regulatory factors. Inclusion of the proline-rich exon 6 results in a Tau protein containing a more rigid and extended hinge region, which might alter microtubule spacing (32). Exon 6 may be involved in modulating the dynamics or extent of the microtubule network. However, it is not clear whether its inclusion in the breast cancer variants has a functional significance. The lack of expression of exon 8 (data not shown) is consistent with the finding that no human tissue has yet been shown to express exon 8. Exon 4A is the longest exon and is exclusively present in the 9-kb Tau transcript. We have shown that exon 4A is expressed in skeletal muscle and heart but not in other types of tissues, including our breast cancer variants (data not shown).

Because Tau overexpression was found in four of our breast cancer taxane-resistant variants, we sought to ascertain the functional role of Tau via knockdown and overexpression experiments. It should be noted that two of these variants also expressed P-gp, and that taxane resistance in the P-gp–positive variants was completely reversed by PSC-833. Because the MCF-7 parental cell line expressed relatively high baseline levels of Tau, we used it for the stable shRNA-mediated Tau silencing experiments. Our results showed that the depletion of Tau did not alter cellular response to taxanes (Fig. 6A). We also knocked down Tau in the human ovarian carcinoma OVCAR-3 cell line, which expresses moderate baseline levels of Tau. We again showed that although Tau was successfully depleted (Fig. 6B), the sensitivity to taxanes was not affected. To confirm our findings, we used eight independent siRNAs including two siRNAs previously published by Rouzier et al. (10). Although we observed >90% Tau silencing, taxane sensitivity was not altered relative to wild-type and nontargeting controls in both the MCF-7 and ZR-75-1 breast cancer cells (Fig. 6D). This is in contrast to other reports—that downregulation of Tau by transient siRNA transfection conferred sensitivity to taxanes in these two cell lines (10, 12).

There are important differences between our set of experiments and those reported by Rouzier et al. (10) and Wagner et al. (12). First, we used both an shRNA approach via lentiviral delivery, which allowed us to select cells with stable Tau knockdown, and a transient siRNA transfection method. Second, our drug exposure was for 72 hours for the SRB cell proliferation assays rather than 48 hours, allowing cells to undergo at least three cell divisions in the presence of the drug. Third, we confirmed our results with a clonogenic assay to assess cell survival after drug exposure rather than the less reliable tetrazolium-based assay or CellTiter-Glo luminescent cell viability assays used in the prior studies.

Tau belongs to the same family of proteins as MAP4; therefore, we hypothesized that perhaps downregulation of Tau is compensated by changes in expression of other MAPs or tubulin proteins. We examined the expression of MAP4, stathmin-1, and tubulin isoforms by Western blot and did not show any significant changes in expression (Fig. 6C). We also measured the kinetics of taxane-driven tubulin polymerization in MCF-7 cells knocked down in Tau, showing no difference in comparison with parental or luciferase shRNA controls (data not shown). Altogether, these results show that depletion of Tau does not confer taxane sensitivity, and suggest that Tau overexpression in taxane-resistant breast cancer clinical specimens may be an epiphenomenon associated with another taxane resistance mechanism(s).

To further evaluate a possible causal relationship between Tau overexpression and taxane resistance, we developed three T-47D clones that stably express Tau 3R, three clones stably expressing Tau 4R, and three clones stably expressing vector alone. We hypothesized that if Tau indeed competes with taxanes for binding to microtubules, then cell lines overexpressing Tau should show resistance to taxanes. Our results show that the overexpression of Tau did not result in resistance to taxanes (Fig. 5A). In fact, some of the clones overexpressing Tau showed slightly more sensitivity to taxanes compared with vector alone.

Tau expression is reported to be regulated by estrogen. The Tau gene contains an imperfect estrogen receptor (ER) response element upstream of its promoter, and Tau expression has been induced in an estrogen-dependent fashion (33, 34). Tau levels were substantially higher in our T-47D/TxTP50 and MDA-MB-231/TxTP50 variants, as measured by quantitative real-time PCR, whereas ER levels did not significantly change from parental cells (data not shown). It was previously suggested that high levels of Tau may be a potential marker of sensitivity to antiestrogen therapy, whereas...
low levels of Tau may be a potential marker of sensitivity to paclitaxel therapy (35). Our findings indicate that upregulation of Tau in taxane-resistant variants is not dependent on upregulation of ER.

A recently published, extensive analysis of Tau expression in the NSABP-B28 study confirmed that high Tau expression was associated with ER expression in breast cancers. However, there was no association of Tau expression and clinical benefit from paclitaxel chemotherapy in either ER-positive or ER-negative patients (36). Thus, Tau expression does not seem to be a useful biomarker for clinical sensitivity to taxanes in breast cancers.

In summary, in this study, we show that modulation of Tau expression (knockdown and overexpression) does not result in altered cellular responses to taxanes in several breast cancer cell lines and one ovarian cancer cell line. These findings indicate that Tau expression is not a mechanism of resistance to taxanes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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