Biomarkers for Sensitivity to Docetaxel and Paclitaxel in Human Tumor Cell Lines In Vitro

ELZBIETA IZBICKA, DAVID CAMPOS, GILBERT CARRIZALES and ANTHONY TOLCHER

Cancer Therapy and Research Center, The Institute for Drug Development, 14960 Omicron Drive, San Antonio, TX 78245, U.S.A.

Abstract. Background: Paclitaxel and docetaxel affect microtubule polymerization, yet surprising differences in tumor sensitivity to the taxanes have been observed. Docetaxel was superior to paclitaxel in inhibiting in vivo growth of human lung and prostate but not breast cancer models. Materials and Methods: We compared drug cytotoxicity, effects on β-tubulin isoforms, markers of apoptosis and proteomic profiles in human prostate (LNCaP), lung (SK-MES, MV-522) and breast (MCF-7, MDA-231) cancer cell lines in vitro. Results: Cytotoxicity followed the order SK-MES< MV-522< LNCaP< MCF-7< MDA-MB-231; docetaxel was more effective. Cytotoxicity directly correlated with Bcl-2 expression in vitro and inversely correlated with docetaxel sensitivity in vivo. Proteomic profiling identified a protein expressed in lung and prostate cells, which was differentially regulated by docetaxel and paclitaxel in SK-MES. Conclusion: The superior activity of docetaxel in tumors with low Bcl-2 warrants further studies on biomarkers for drug sensitivity and investigation of docetaxel in combination with drugs that reduce Bcl-2 gene expression.

The anticancer activity of the taxanes docetaxel and paclitaxel has been attributed, in part, to the shared ability to stabilize microtubules. Yet, significant differences in efficacy were also reported. The drugs differently affect the cycle phases and are known to be hydroxylated in liver by different cytochrome P-450 (CYP) forms. Docetaxel has higher affinity for microtubules than paclitaxel and more potently induces Bcl-2 phosphorylation (1). Furthermore, differences in cell retention times and pharmacokinetic properties may explain the more favorable benefit-to-risk ratio for docetaxel as a single agent and in combination therapy (2).

Little is known about predictive biomarkers for tumor sensitivity to docetaxel and paclitaxel. The resistant phenotype of human prostate cell lines was associated with transient expression of β-tubulin isoforms (3). The class II β-tubulin isotype was a marker of docetaxel activity in advanced breast cancer (4). Apoptosis and Bcl-2 levels correlated with breast cancer response to docetaxel/doxorubicin chemotherapy (5), and patients with breast cancer whose tumors did not express Bcl-2 had a better pathological response (6). The discovery of predictive biomarkers for drug response will increasingly rely on methods that allow for global screens of whole tumor and/or host proteomes (7-12). Despite considerable progress in the application of proteomics to the early detection of human cancers, chemotherapy-induced changes in the tumor or host proteome have not been extensively examined to date (7, 13, 14).

Our long-term goal is to identify predictive biomarkers for docetaxel and paclitaxel sensitivity. To date, intriguing differences have been observed in the sensitivity of human tumor xenografts to docetaxel and paclitaxel administered at the optimal doses and schedules in LNCaP prostate, SK-MES and MV-522 lung and MCF-7 and MDA-MB-231 breast tumor models. Docetaxel demonstrated a statistically significant activity, which was superior to paclitaxel in the LNCaP, SK-MES and MV-522 models. In MCF-7 xenografts, docetaxel was only mildly active and paclitaxel was toxic at the high and inactive at the low dose. While docetaxel was more active than paclitaxel in the lung and breast tumor models, paclitaxel was more active against MDA-231 (15). The present study examined the mechanistic reasons behind differential sensitivity of human tumor models to docetaxel and paclitaxel. We used the same human tumor models in vitro to investigate the effects of the taxanes on expression of select biomarkers implicated as downstream; drug targets β-tubulin isoforms and markers of apoptosis or survival (Bcl-2, Bcl-XL, Bax, survivin).
addition, global proteomic profiles were compared in cells treated with the drugs versus vehicle control to identify specific protein clusters, which might correlate with the drug sensitivity. The study demonstrated the superior activity of docetaxel in tumor cell lines with low levels of Bel-2, and identified protein species that were uniquely expressed in lung and prostate tumor models characterized by high sensitivity to docetaxel.

Materials and Methods

Reagents and cell lines. Docetaxel (Taxotere®) was provided by Aventis. The drug was dissolved in 70% ethanol to prepare a 1 mM stock solution. The final test solutions contained 0.1% ethanol. Paclitaxel was purchased from Sigma Chemical Company (St. Louis, MO, USA). A 10 mM stock solution of paclitaxel was prepared in 100% DMSO, and all dilutions contained 0.1% DMSO.

LNCaP prostate, SK-MES lung and MCF-7 and MDA-MB-231 breast carcinoma cell lines were obtained from the American Type Cell Collection (Rockville, MD, USA). The MV-522 lung carcinoma cell line was obtained from a culture maintained at the Institute for Drug Development. The cells were cultured in RPMI medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco) and propagated at 37°C in a humidified atmosphere containing 5% CO2. Exponentially growing cells in 100 μl of cell culture medium were plated at different densities ranging from 1x10³ to 1.2x10⁴ cells/well on day 0 in 96-well microtiter plates to determine doubling times.

Growth inhibition assay. The cells were plated on day 0 into 96-well microtiter plates at a final density of 2x10³ cells/well for the 3-day incubation or 10⁴ cells/well for the 5-day incubation and allowed to attach for 24 hours prior to drug exposure on day 1. The cells were incubated with the drugs for 3 or 5 days, then 100 μl of growth medium was removed and the cells incubated with 20 μl MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium, inner salt; MTS] at 1.9 mg/ml in phosphate-buffered saline (PBS) pH 6.0 for 1 hour at 37°C (16). The absorbance of the soluble formazan salt was measured in a Dynex HD microplate reader at 490 nm and the IC₅₀ values calculated using four-parameter fit.

Drug treatment. Based on the respective IC₅₀ values, subtoxic concentrations of docetaxel (500 pM for all cell lines in 3- and 5-day exposures, except for 50 pM docetaxel in MDA-MB-231 for a 5-day treatment) and paclitaxel (1000 pM for all cell lines in 3- and 5-day exposures, with the exception for 500 pM paclitaxel in MDA-MB-231 for a 5-day treatment) were selected. To investigate the concentration-dependence of the biomarker expression, the cell lines were cultured for 3 days with three concentrations of the taxanes, corresponding to IC₂⁵, IC₅₀ and IC₇⁵ values (Table I). The cell seeding density for the 3-day tests was 4x10³ cells/well, and 2x10⁴ cells/well for the 5-day cell treatment. The cells were plated in 6-well plates (Falcon, Fisher Scientific) on day 0 and cultured in the presence of the drugs or the vehicle alone as a control added once on day 1, and harvested on days 3 and 5 for measurements of biomarker expression. At the time of the harvest, the cells were washed with PBS pH 7.4, collected by trypsinization, and washed again in PBS by centrifugation in 15 ml conical tubes. Floating cells were removed from the media by centrifugation and pooled with the trypsinized cells. Combined cell harvests were suspended in 1 ml of PBS, transferred to Eppendorf tubes and centrifuged again. The pellets were lysed in osmotic lysis buffer and the protein concentration determined as described (17).

Western blots. Protein lysates normalized to an equal amount (50 µg) of protein were processed and analyzed as described (17). Western blots were probed with anti-β-actin ascites IgM (Oncogene Research Products, Boston, MA, USA) or the following monoclonal murine anti-human antibodies: anti-β-tubulin ascites IgG1 cross-reactive with all β-tubulin subtypes (Sigma Chemical Co.); anti-βII tubulin IgG2b (cross-reactive with β-tubulin I and II subtypes, and anti-β III tubulin IgG2b (InnoGenex, San Ramon, CA, USA), anti-Bcl-2 IgG1, anti-Bcl-Xl and anti-Bax (Dako, Carpinteria, CA, USA). Anti-survivin was obtained from Santa Cruz (Santa Cruz, CA, USA). Incubations with the primary antibodies were done overnight at 4°C. Anti-mouse IgG horseradish peroxidase conjugate (Cell Signaling, Beverly, MA, USA) was used as the secondary antibody. The chemiluminescence was captured, quantified and normalized for β-actin expression (17).

SELDI MS-TOF. Protein lysates were processed robotically on a Biomek 2000 liquid handling system in a 96-well format for SELDI analysis (Biomek 2000; Beckman Coulter, Fullerton, CA, USA). Briefly, 20 µL aliquots of cell lysates adjusted to a final protein concentration of 1 mg/mL were pretreated with 8 M urea and 1% CHAPS and vortexed for 10 minutes at 4°C. A further dilution was made in 1 M urea, 0.125% CHAPS and PBS. Each sample position was randomized and spotted in duplicate onto copper-coated im mobilized metal affinity (IMAC-Cu) ProteinChips in a bioprocessor. The ProteinChips were then incubated at room temperature for 30 minutes, followed by washes with PBS and water. The IMAC-Cu chip arrays were allowed to air dry, and a saturated solution of sinapinic acid in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was added to each spot. Other arrays tested included SAX-2 and WCX-2. The protein chip arrays were analyzed with the SELDI ProteinChip System (PBS-II, Ciphergen Biosystems, Fremont, CA, USA). The spectra were generated by the accumulation of 192 shots at laser intensity 220 in a positive mode. The protein masses were calibrated externally with the use of purified peptide
standards. Afterward, each protein peak was labelled and its intensity was normalized for total ion current to account for variation in ionization efficiencies.

SELDI data analysis. Peak clustering, i.e., creation of peaks of similar mass that are treated as the same substance across multiple spectra, was performed with Biomarker Wizard and Ciphergen Express Software (Ciphergen Biosystems) at settings that provide a 20% minimum peak threshold, 0.3% mass window and 3 to 2 signal/noise determination. The peak intensities from duplicate samples were then averaged. The p values were calculated to test the null hypothesis that the median of the peak intensities of the groups are equal. Paired statistics was used to calculate the Wilcoxon signed-rank test. The cluster plots by M/Z, heat maps and hierarchical clustering were generated. The color and intensity of each cell was determined by the log normalized intensity, which was defined by log normalized intensity = log intensity – log average intensity. The log normalized intensities were further divided by the largest absolute log normalized intensity in the map. The quotients were finally used to map the positive values to red and negative values to green, indicative of the positive or negative differences from the average peak intensity, respectively.

Results

Cytotoxicity. Since the biological activity of taxanes is schedule dependent and increases over time (2, 18, 19), we evaluated the cytotoxicity of docetaxel and paclitaxel following a 3- and 5-day cell exposure. As shown in Figure 1, the IC50 values for docetaxel spanned ~3-fold concentration range and decreased over time in all models. After a 3-day treatment, SK-MES (IC50 of 1,576 pM) was the least sensitive and MDA-MB-231 (IC50 of 499 pM) was the most sensitive to docetaxel. At day 5, the cytotoxicity of docetaxel increased over 2-fold in all models except MDA-MB-231, where the drug was more toxic (IC50=35 pM). Paclitaxel was approximately 2-fold less toxic than docetaxel, although a similar trend of cytotoxicity was noted. SK-MES (IC50=3,801 pM) was the least sensitive and MDA-MB-231 (IC50=933 pM) was the most sensitive to paclitaxel. The cytotoxicity of paclitaxel did not increase greatly by day 5 in LNCaP, MCF-7 and MV-522. In MDA-MB-231 and SK-MES, paclitaxel toxicity at day 5 increased about 2-fold in comparison with day 3. Notably, steep growth inhibition curves were similar in all models except MDA-MB-231, indicating a possibility for a different mechanism of action. Overall, the cytotoxicities of docetaxel and paclitaxel were lowest in the lung, followed by the prostate and breast cancer models. The tumor models characterized by low cytotoxicity of docetaxel in vitro, also responded better to the drug in vivo.

Biomarker effects. To mimic experimental conditions from the in vitro studies that were performed at single optimal doses of docetaxel and paclitaxel (15), single subtoxic concentrations of docetaxel and paclitaxel were selected to evaluate drug effects on biomarker expression. Evaluation of basal levels of biomarkers in cells treated with the vehicle has shown that the expression of β-tubulin isoforms, Bcl-XL and Bax was comparable in all cell lines. Survivin was undetectable in SK-MES and MV-522 (data not shown). A large variation in Bcl-2 expression was noted. The protein was not detectable in SK-MES under the experimental conditions used for immunoblots for other cell lines. Even when the protein load and antibody concentrations were increased, Bcl-2 was barely detectable. These data indicate a significantly lower level of Bcl-2 in SK-MES than in other cell lines. Low Bcl-2 was also seen in MV-522; it was intermediate in LNCaP and high in the two breast cancer cell lines. Figure 2 illustrates the quantitative analysis of Bcl-2 expression normalized to the MCF-7 levels (defined as 100%). Interestingly, these results
coincided with the tumor xenograft sensitivity to docetaxel.

Figure 3 shows representative immunoblots probed for select biomarkers in cell lysates obtained after cell treatment with the subtoxic concentrations of docetaxel and paclitaxel, or the vehicle controls. The expression of β-actin was uniform in all groups, consistent with comparable levels of total protein. Drug treatment did not affect survivin in any model (data not shown). In breast cancer models, MCF-7 probed for expression of β tubulin isotypes with the panning antibody or the antibodies cross-reactive with the βII and
Figure 4. Cluster analysis on WCX array.
βIII isotypes did not reveal significant differences between paclitaxel or docetaxel versus the vehicle. Docetaxel down-regulated Bcl-2 at day 3 and Bcl-X<sub>L</sub> at day 5. Both taxanes slightly up-regulated Bax at day 3. In MDA-MB-231, docetaxel transiently inhibited pan-tubulin at day 3. The levels of βII<sub>H</sub> tubulin were not affected by either drug. Small up-regulation of βII<sub>H</sub> tubulin occurred after docetaxel treatment for 5 days. Docetaxel inhibited Bcl-2 and Bcl-X<sub>L</sub> at day 3, but Bax was not affected by either drug.

In LNCaP, β-tubulin expression assessed with the panning antibody was comparable in all treatment groups. The levels of βII<sub>H</sub> tubulin increased with increased sensitivity to docetaxel. In the lung cancer models, the pan-tubulin expression in MV-522 was stable under all conditions. Both βI<sub>H</sub> and βII<sub>H</sub> tubulin were up-regulated by paclitaxel and docetaxel for 3 days and by docetaxel for 5 days. Bcl-2 was inhibited by treatment with both taxanes for 3 days, and after cell exposure to docetaxel for 5 days. Bcl-X<sub>L</sub> expression was comparable under all conditions. In SK-MES, pan-tubulin and βII<sub>H</sub> tubulin were down-regulated by docetaxel after 3 days. No changes in βII<sub>H</sub> tubulin were observed. Bcl-2, Bcl-X<sub>L</sub> and Bax levels were not affected by any drug treatment.

**SELDI TOF-MS.** These experiments were performed to determine if there are tumor-specific proteins whose expression correlated with increased sensitivity to docetaxel. For identification of drug-specific protein clusters, cells were treated for 3 days with three concentrations of the taxanes, corresponding to IC<sub>25</sub>, IC<sub>50</sub> and IC<sub>75</sub>. Proteomic profiling analysis was performed on three different ProteinChip affinity surfaces to simplify the proteome and profiling analysis was performed on three different ProteinChip affinity surfaces to simplify the proteome and improve cluster identification. Protein profiles on the strong anion exchanger SAX2 were very similar for both drugs. Capture of proteins on the metal affinity surface IMAC-3-Cu resulted in clearly differential profiles, but the differences between docetaxel and paclitaxel were not statistically significant (data not shown). In contrast, there were major differences in protein profiles in a weak cation exchange array WCX2. Corresponding heat maps of the protein clusters are shown in Figure 4. In SK-MES, 26 proteins were identified as up-regulated or down-regulated after drug treatment in comparison with the respective vehicle controls. Two protein peaks at M/Z 4270 and M/Z 5600 showed the significantly different drug effects (p=0.05). These proteins were down-regulated by docetaxel and up-regulated by paclitaxel in a concentration-dependent mode. The peak at M/Z 4270 was also detectable in MV522, where it was also (albeit not-significantly) up-regulated by paclitaxel. The second peak at M/Z 5600 was not detected in MV522. In LNCaP, the peak at M/Z 4270 showed some concentration dependence on docetaxel, but the second peak was not affected by either drug. These two peaks were not affected by docetaxel or paclitaxel in the two breast cancer cell lines.

**Discussion**

The differential activities of docetaxel and paclitaxel observed in preclinical and clinical studies cannot be fully explained based on pharmacological data alone. The search is under way to identify prognostic biomarkers of drug efficacy. Our earlier work pointed to marked differences in the activities of docetaxel and paclitaxel in human breast, prostate and lung xenograft models, and prompted us to further investigation of predictive biomarkers for the drugs in the same models in vitro. Low toxicity of docetaxel in vitro, which translated to better sensitivity to the drug in human tumor xenografts, suggests that the efficacy in vivo is not a function of non-specific toxicity, but reflects a more targeted, tumor-specific mechanism of action.

This study demonstrated cell-specific and time-dependent effects of docetaxel and paclitaxel on the expression of β-tubulin isotypes. Although the effects were not tumor-specific, the effects of the taxanes on β-tubulin isotypes deserve more attention. Taxane resistance was shown to correlate with different expression patterns of β-tubulin isotypes (20, 21). It is of interest if modulation of β-tubulin expression could be amplified upon long-term drug treatment and paralleled by emerging drug-resistant phenotype. Drug treatment may affect the subcellular localization of β-tubulin isotypes. Unusual nuclear localization of βII<sub>H</sub>-tubulin was first described in cancer cells (22) and in human tumor specimens (23). Treatment with paclitaxel was shown to increase the amounts of nuclear α- and βII<sub>H</sub>-tubulin in human cancer cells (24).

Although the effects of taxanes on Bcl-X<sub>L</sub>, Bax and survivin did not correlate with specific tumor types, model-specific effects on Bcl-2 were notable. Docetaxel inhibited Bcl-2 more effectively than paclitaxel in four of the five cell lines, and the cellular expression of Bcl-2 apparently correlated with the potency of docetaxel in vivo. The data suggest that the Bcl-2 status might be an important determinant of docetaxel sensitivity. Earlier studies have shown that docetaxel was highly active against the DU-145 tumor xenograft model and that by Western blot analysis no expression of Bcl-2 was found in the cells before or following in vivo treatment (25). Our data are consistent with that report and further underscore that tumor models with low levels of Bcl-2 may demonstrate superior response to docetaxel by an as yet unidentified mechanism.
With recent advances in mass spectrometry, investigation of proteins over a wide range of molecular weights in biological specimens is feasible. Mass spectrometry protein profiling has been focused mainly on disease markers, not pharmacoproteomic determination of predictive biomarkers of drug response. This study suggests that biomarkers for docetaxel sensitivity can be identified by a global proteomic screening. The finding of a single protein species as a putative biomarker of drug response is consistent with limited reports to date. Very low numbers of disease-specific markers were identified in ovarian and other cancers (26-30). Although the human plasma proteome is thought to contain over 500,000 proteins and represents a significantly richer proteome than the cell extracts, only a single protein peak of M/Z 2790 was identified in plasma from patients with breast cancer that was induced by paclitaxel and, to a lesser extent, by a combination chemotherapy (14).

Yet, drug treatment is likely to affect the expression of multiple proteins, some of which may belong to the "deep proteome", i.e., represent proteins of low abundance. Identification of "deep proteome" biomarkers may be challenging even with advanced mass spectrometry tools. The limitations of detection may be difficult to overcome when small clinical specimens are used for biomarker discovery. Thus, preclinical in vitro and in vivo model systems offer unique opportunities for the discovery, identification and extensive characterization of pharmacoproteomic biomarkers, which ultimately will be validated in clinical studies.

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Received June 13, 2005
Accepted June 21, 2005