Antiproliferative Mechanism of Action of the Novel Taxane Cabazitaxel as Compared with the Parent Compound Docetaxel in MCF7 Breast Cancer Cells

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Abstract

Cabazitaxel, a novel chemotherapeutic taxane, is effective against docetaxel-resistant cells and tumors. It is approved for treatment of metastatic hormone-refractory prostate cancer in patients pretreated with docetaxel. Objective responses have been observed in many other cancers, including pretreated metastatic breast cancer. Cabazitaxel and docetaxel share a high degree of structural similarity. The basis for cabazitaxel’s efficacy is unclear, and its mechanism has not been described. We compared the effects of cabazitaxel and docetaxel on MCF7 human breast cancer cells expressing fluorescent tubulin. Both drugs inhibited cell proliferation (IC50s, cabazitaxel, 0.4 ± 0.1 nmol/L, docetaxel, 2.5 ± 0.5 nmol/L) and arrested cells in metaphase by inducing mitotic spindle abnormalities. Drug concentrations required for half-maximal mitotic arrest at 24 hours were similar (1.9 nmol/L cabazitaxel and 2.2 nmol/L docetaxel). Cabazitaxel suppressed microtubule dynamic instability significantly more potently than docetaxel. In particular, cabazitaxel (2 nmol/L) suppressed the microtubule shortening rate by 59% (compared with 49% for 2 nmol/L docetaxel), the growing rate by 33% (vs. 19%), and overall dynamicity by 83% (vs. 64%). Cabazitaxel was taken up into cells significantly faster than docetaxel, attaining an intracellular concentration of 25 μmol/L within 1 hour, compared with 10 hours for docetaxel. Importantly, after washing, the intracellular cabazitaxel concentration remained high, whereas the docetaxel concentration was significantly reduced. The data indicate that the potency of cabazitaxel in docetaxel-resistant tumors is due to stronger suppression of microtubule dynamics, faster drug uptake, and better intracellular retention than occurs with docetaxel. Mol Cancer Ther; 13(8): 2092–103. ©2014 AACR.

Introduction

The chemotherapeutic taxanes paclitaxel (Taxol) and docetaxel (Taxotere) play important, well-established roles in the treatment of a variety of malignancies, including a broad spectrum of solid tumors (1, 2). Paclitaxel is widely used for the treatment of breast, ovarian, and non–small cell lung cancer, whereas docetaxel-containing regimens are indicated for metastatic prostate, breast, and other cancers (3, 4). Despite the clinical success of microtubule-targeting agents, toxicity (mostly neutropenia and neurotoxicity), complex formulations (use of Cremophor EL), and limited oral bioavailability restrict their clinical use in cancer therapy (1). Many tumor types are refractory and/or develop acquired resistance to these drugs (5–7). Paclitaxel and docetaxel both have a high affinity for multidrug resistance proteins (7–9).

Cabazitaxel (Jevtana) is a novel third-generation semisynthetic analog of docetaxel (8, 10–12). Structurally, cabazitaxel and docetaxel are very similar with the exception of 2 methoxy side chains in cabazitaxel that substitute for hydroxyl groups in docetaxel (Fig. 1A). In preclinical testing, cabazitaxel demonstrated activity in both docetaxel-sensitive and docetaxel-resistant cancers (13, 14). In addition, the terminal half-life for cabazitaxel in humans is 95 hours (15) as compared with 12 hours for docetaxel (16). Cabazitaxel was approved in 2010 as a second-line treatment in men with metastatic castration-resistant prostate cancer that failed docetaxel-containing regimens (17, 18). Like paclitaxel and docetaxel, cabazitaxel stabilizes microtubules against cold-induced depolymerization and promotes assembly of tubulin into microtubules (12, 14).

Microtubules are major components of the eukaryotic cytoskeleton. They are involved in cell division, migration, signaling, and intracellular trafficking and are important in cancer cell proliferation and metastasis (2). Microtubules are composed of αβ-tubulin heterodimers that link together noncovalently during polymerization to
**Figure 1.** Concentration-dependent effects of cabazitaxel and docetaxel (A) on inhibition of proliferation (B), induction of cell death (C), mitotic arrest (D), and G2–M arrest (E and F) in MCF7 cells. A, the chemical structures of docetaxel (Taxotere) and cabazitaxel (Jevtana). In cabazitaxel methoxy side chains substitute for hydroxyl groups in docetaxel (highlighted in red). B, cells were incubated with cabazitaxel (CBZ) or docetaxel (DOC) for 72 hours, and inhibition of proliferation was measured using a sulforhodamine B assay. Cabazitaxel and docetaxel inhibited cell proliferation with IC50 values of 0.4 ± 0.1 (SEM) nmol/L and 2.5 ± 0.5 (SEM) nmol/L, respectively. C, cells were incubated with vehicle control (x), cabazitaxel for 24 (CBZ-24 h), 48 (CBZ-48 h), and 72 (CBZ-72 h) hours, and docetaxel for 24 (DOC-24 h), 48 (DOC-48 h), and 72 (DOC-72 h) hours, and the population of combined apoptotic/dead cells was determined by flow cytometry. Controls at 24, 48, and 72 hours displayed similar levels of apoptosis (1.5%, –x–). D, cells were incubated with cabazitaxel (CBZ) and docetaxel (DOC) for 24 hours, and the percentage of mitotic cells was determined using immunofluorescence microscopy. Cabazitaxel and docetaxel induced mitotic arrest with IC50’s of 1.9 and 2.2 nmol/L, respectively. Cells were incubated with cabazitaxel (CBZ) for 24 (CBZ-24 h), 48 (CBZ-48 h), and 72 (CBZ-72 h) hours, and with docetaxel (DOC) for 24 (DOC-24 h), 48 (DOC-48 h), and 72 (DOC-72 h) hours, and the population of G2–M cells was determined by flow cytometry. Control (CTR) cells in G2–M at 24 (CTR-24 h), 48 (CTR-48 h), and 72 hours gray diamond. Results are means and SEM of at least 3 independent experiments. **P < 0.01; ***P < 0.001 by the Student t test.
form 25-nm-diameter hollow cylindrical filaments (19). Microtubules are highly dynamic structures that alternate between periods of growth and shortening through the addition or loss of tubulin subunits at microtubule ends (20). This nonequilibrium behavior is termed “dynamic instability” and occurs both in vitro with microtubules reassembled from purified tubulin and in cells. Dynamic microtubules are especially important during mitosis when they are required for the capture and complex movements of chromosomes, including chromosome alignment during metaphase and separation during anaphase (21, 22). Dynamic spindle microtubules are crucial to successful cell division, making cells highly susceptible to drugs that suppress microtubule dynamic instability at low concentrations (23, 24).

The taxanes, including paclitaxel and docetaxel, are microtubule-stabilizing antitumor drugs that enhance microtubule polymerization at high concentrations (25, 26). All taxanes bind to the same or to an overlapping taxoid-binding site on β-tubulin, located on the inner surface of the microtubule (27, 28). Taxanes and other microtubule-targeting drugs inhibit cancer cell proliferation by binding to microtubules and suppressing microtubule dynamics during the particularly vulnerable mitotic stage of the cell cycle without significantly altering the mass or organization of microtubules (26). Stabilization of mitotic spindle microtubule dynamics leads to mitotic arrest, inhibition of cell proliferation, and cell death (23, 24).

The basic mechanisms of action of cabazitaxel on microtubules have not been elucidated. It is especially important to understand the differences in the mechanisms that may be responsible for cabazitaxel’s efficacy in docetaxel-refractory cancers (17). We used EGFP-α-tubulin-expressing MCF7 human breast cancer cells to compare the concentration- and time-dependent effects of docetaxel and cabazitaxel on cell proliferation, microtubule arrangement and dynamic instability, mitosis, apoptosis, and drug uptake and retention. We have previously used EGFP-α-tubulin-expressing MCF7 cells extensively for determination of drug effects on microtubule dynamic instability (29–32) because they are flat enough to enable tracing the length changes of individual microtubules. In addition, both docetaxel and cabazitaxel are effective in breast cancer singly or in combination (10, 33).

In comparing cabazitaxel and docetaxel, we found that both drugs inhibit cell proliferation and induce mitotic arrest in association with suppression of microtubule dynamic instability. However, cabazitaxel suppresses microtubule dynamic instability significantly more potently than docetaxel and induces a more sustained G2-M arrest over 72 hours. The rate of cabazitaxel’s uptake into cells is significantly faster than that of docetaxel, and the intracellular concentration of cabazitaxel does not change significantly upon washing the cells, whereas docetaxel levels are significantly reduced. Together, these findings suggest that cabazitaxel’s significantly more effective suppression of microtubule dynamics, rapid intracellular uptake, and prolonged drug retention play significant roles in its efficacy in docetaxel-resistant tumors.

Materials and Methods

Materials

Chemicals and materials were from Sigma-Aldrich unless otherwise noted. Cabazitaxel and docetaxel were provided by Sanofi-Aventis (France) and stored as aliquots of 10 mmol/L stock solutions in DMSO at −20°C. Stock solutions were diluted in DMSO to 1 mmol/L and further diluted in cell culture medium.

Cell culture

MCF7 human breast adenocarcinoma cells were cultured in DMEM with 10% fetal bovine serum (Atlanta Biologicals, Inc.), 1% final concentration of nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C, 5.5% CO2. Shortly after purchase (ATCC, 2007), the cells were transfected with an enhanced green fluorescent protein EGFP-α-tubulin vector (Clontech Laboratories; ref. 34). The EGFP-α-tubulin-expressing MCF7 cells were indistinguishable from unmodified MCF7 cells except for their fluorescent microtubules and their doubling time of 35 hours, which was 20% slower than unmodified MCF7 cells. They continue to express EGFP-α-tubulin, were used for all experiments, were free of mycoplasma, and are referred to hereafter as MCF7 cells.

Cell proliferation

Proliferation was measured by a modified sulforhodamine B assay (32). MCF7 cells were seeded in medium (5,000 cells/200 μL) in 96-well plates and incubated 24 hours. Fresh medium with or without the drugs was added, and incubation continued for 72 hours. Cells were fixed, stained, and the optical density determined (490 nm; Victor®V Wallac 1420 Spectrophotometer, PerkinElmer). Triplicates of each condition were tested in each experiment. Results are mean and SEM of at least 4 experiments. The inhibitory concentrations that inhibited cell proliferation by 50% were calculated using Prism 4.0 software (GraphPad Software, Inc).

Cell viability and apoptosis/cell death

Cells were seeded (6 × 104 cells/2 mL) in 6-well plates (24 hours), and incubated with drug for 24, 48, and 72 hours. For viability, all cells were harvested and stained with ViaCount DNA binding dyes (5 minutes; EMD Millipore) and analyzed by flow cytometry (EasyCyte flow cytometer; Guava Technologies, Inc.) to distinguish live and apoptotic/dead cells. For cell-cycle analysis, floating and adherent cells were collected, permeabilized with ice-cold 70% ethanol, washed with PBS, and stained with cell-cycle reagent (EMD Millipore). The DNA content of ≥5,000 cells was measured by flow...
cytometry and analyzed with ModFit LT software (Verity Software House). Results are mean ± SEM, ≥3 independent experiments.

Mitotic arrest
MCF7 cells were seeded as described above for cell viability and incubated with drug for 24 hours; both floating and attached cells were collected and fixed in 3.7% formaldehyde (30 minutes), followed by cold methanol (10 minutes; ref. 29). Fixed cells were mounted on glass slides with antifade agent Prolong Gold-DAPI (Life Technologies, Inc.) to visualize DNA and examined by fluorescence microscopy (Nikon Eclipse E800, ×40 objective). Mitotic cells were rounded cells with condensed chromosomes and no nuclear envelope. The percentage of mitotic cells was determined by counting ≥500 cells for each condition. Results are mean ± SEM, ≥3 experiments.

Immunofluorescence microscopy
Cells were seeded as described above for cell viability on poly-L-lysine-treated coverslips, incubated with drug 24 hours, fixed with 3.7% formaldehyde followed by cold methanol (29) and stained with mouse monoclonal α-tubulin antibody (1:1,000 DM1A) and FITC-conjugated goat anti-mouse antibody (1:1,000, Cappel MP Biochemicals). Centrosomes were stained with rabbit polyclonal anti-pericentrin antibody (1:500, AB4448; Abcam) and rhodamine-conjugated goat anti-rabbit antibody (1:500, Cappel). Cells were mounted as above and imaged using a spectral confocal Olympus Fluoview1000 microscope (PLV1000/S, ×60 oil, N.A. 1.4 objective; Olympus).

Live cell analysis of microtubule dynamic instability
Cells were seeded on coverslips in 6-well plates (3 × 10^4 cells/mL, 2 mL/well) for 24 hours, and then medium was replaced with fresh medium containing drug. Time-lapse images (38 frames/4-second intervals/1 hour at 37 ± 1°C) were taken with a ×100 objective on a Nikon Eclipse E800 microscope equipped with a CoolSNAP HQ2 camera (Roper Scientific GmbH), MetaMorph 4.6 software (Molecular Devices; ref. 35).

Changes in length of individual microtubules were graphed versus time as “life history” plots. Microtubule dynamics parameters were analyzed with IGOR Pro 6.0 (32). Changes ≥0.5 μm were growth or shortening events, <0.5 μm were pause (attenuation). Time-based catastrophe frequency is the number of catastrophes (transitions from growth or pause to shortening) divided by total time spent growing and paused. Rescue frequency is the number of transitions from shortening to growth or pause divided by total time spent shortening. Dynamicity is total length grown and shortened divided by total duration of imaging a microtubule. Results are mean ± SEM, ≥3 experiments.

Uptake of [14C]-cabazitaxel and [14C]-docetaxel
To determine intracellular drug concentration (36), MCF7 cells were seeded into poly-L-lysine-treated scintillation vials (1 × 10^5 cells, 2 mL). After 24 hours, media was replaced with fresh media containing 50 nmol/L [14C] cabazitaxel or 50 nmol/L [14C] docetaxel (final specific activity: 83–86 mCi/mM/L; Sanoﬁ-Aventis Deutschland GmbH, Germany) or unlabeled drug. Media was removed 30 minutes to 20 hours after drug addition, vials rinsed twice with 1 mL PBS, and Ready Protein (Beckman Coulter, Inc.) added for scintillation counting (LS 6500 Multi-Purpose Scintillation Counter; Beckman Coulter, Inc.). Intracellular drug concentration = (moles of intracellular drug bound/mean cell volume) × (the number of cells per vial). Mean cell volume (4.57 × 10^-12 L) is the mean diameter of cells rounded by trypsinization (n = 30). After 20 hours drug, some cell vials were washed (2 washes fresh media, 30 minutes/wash) and intracellular drug concentration was determined 1 to 20 hours after wash. All time points were measured in duplicate. Results are means ± SEM of ≥3 experiments.

Results
Cabazitaxel and docetaxel inhibit MCF7 cell proliferation and arrest cells in mitosis
The effects of cabazitaxel and docetaxel on proliferation of MCF7 cells were determined after 72 hours incubation (Fig. 1B). Both taxanes inhibited proliferation with cabazitaxel exhibiting greater potency than docetaxel (IC50’s ± SEM, 0.4 ± 0.1 nmol/L and 2.5 ± 0.5 nmol/L, respectively, P < 0.05). Minimal inhibition (9%) occurred at 1 nmol/L cabazitaxel or docetaxel, and approximately 80% inhibition occurred at 1,000 nmol/L for each drug (Fig. 1B).

The numbers of viable, and apoptotic/dead MCF7 cells induced by cabazitaxel and docetaxel (0.1–100 nmol/L) at 24, 48, and 72 hours of incubation were determined by flow cytometry. Both cabazitaxel (dashed lines, open symbols) and docetaxel (solid lines, filled symbols) induced time- and concentration-dependent apoptosis (Fig. 1C). At 24 hours (lowest curves), cabazitaxel (open squares) and docetaxel (filled squares) induced similar numbers of apoptotic/dead cells at all concentrations tested. For example, cabazitaxel and docetaxel (100 nmol/L) induced 3.5% and 4% apoptotic/dead cells, respectively, compared with 1.5% in controls (Fig. 1C). With increased time of incubation, cabazitaxel induced significantly more cell death than docetaxel at all concentrations tested. For example, at 72 hours, 100 nmol/L cabazitaxel induced 37% apoptotic/dead cells as compared with 27% for 100 nmol/L docetaxel (P < 0.05; Fig. 1C).

To examine whether the antiproliferative effects were associated with mitotic arrest, we counted mitotic cells at 24 hours incubation with a range of drug concentrations. Only 2.3% of control cells were mitotic (Fig. 1D). Half-maximal mitotic arrest occurred at 1.9 nmol/L cabazitaxel (squares) and 2.2 nmol/L docetaxel (diamonds). At high drug concentrations (10–1,000 nmol/L), the number of mitotically-arrested cells at 24 hours was also similar for both drugs, reaching 60% to 65% (Fig. 1D).
We also determined the drug effects on G2–M arrest. G2–M arrest measures the number of cells in G2, in M, and also cells that have slipped out of mitosis without undergoing cytokinesis or division. After 24 hours, both drugs induced similar G2–M levels but their effects diverged after longer drug incubation. By flow cytometry cells in G2–M increased from 25% in controls to 84% to 88% at 10 to 100 nmol/L of either drug at 24 hours (Fig. 1E and F). Interestingly, with cabazitaxel the high degree of G2–M arrest was sustained with longer incubation times, but docetaxel did not maintain the same high level of G2–M arrest at 48 and 72 hours. For example, in the presence of 10 and 100 nmol/L docetaxel, the number of cells in G2–M decreased from 84% at 24 hours to 61% at 72 hours.

**Cabazitaxel and docetaxel induced similar changes in microtubule organization**

MCF7 cells were incubated with a range of drug concentrations for 24 hours, fixed, and stained for microtubules, centrosomes, and DNA and examined by immunofluorescence microscopy. In control interphase cells microtubules (stained green) were fine filaments throughout the cell including the cell periphery, with a pair of centrosomes (red) next to the nucleus (Fig. 2A). At cabazitaxel and docetaxel concentrations that induced half-maximal mitotic arrest (2 nmol/L), most interphase cells were similar to controls, although a few were slightly rounded (Fig. 2B and C). At 10 nmol/L of either drug (approximately 5 × IC50), microtubules were bundled in...
the interphase cell center and were shorter, more curved, and fragmented at the cell periphery than in controls (not shown). At 100 nmol/L cabazitaxel or docetaxel, most cells were rounded, and fragmented or curved microtubules at the cell periphery increased, as did prominent microtubule bundles in the cell center (Fig. 2D and E). Centrosome pairs were localized in the cell periphery. Both drugs induced some giant multinucleated cells and apoptotic figures (Fig. 2D and E).

Drug effects on mitotic spindle organization at 24 hours are shown in Fig. 2F–L. Control mitotic cells had well-separated centrosomes and well-formed bipolar spindles with chromosomes in a compact central metaphase plate (Fig. 2F) and duplicated centrosomes at opposite spindle poles. Both drugs induced spindle abnormalities at concentrations that induced little or no detectable change in the interphase microtubule network (1 nmol/L, 0.5 × IC50 for mitotic arrest for both drugs; Fig. 2G and H). A few cells had normal bipolar spindles with compact metaphase plates of chromosomes (not shown). Most cells had abnormal spindles (37), with more and longer astral microtubules than in controls, with from few to many uncongressed chromosomes remaining at the spindle poles rather than forming a well-defined metaphase plate (Fig. 2G and H, arrowheads). At 1 × IC50 (2 nmol/L), both drugs induced bipolar spindle abnormalities (Fig. 2I and J), and multipolar spindles were common (Fig. 2I and J, arrows). At 1 to 2 nmol/L of both drugs, a few mitotically arrested cells had abnormal monoastral ball-shaped spindles, with little, if any, centrosome separation. At 5 to

Figure 3. Dynamic instability of microtubules in live interphase MCF7 cells incubated with cabazitaxel or docetaxel. A, time-lapse images of individual microtubules in the lamellar periphery of cells were recorded by video epifluorescence microscopy: control (top row), cell incubated with 2 nmol/L cabazitaxel (middle row), and cell incubated with 2 nmol/L docetaxel (bottom row), at 24 hours. Microtubule ends (arrows) were tracked over time to produce life history plots (changes in length of individual microtubules over time) shown in B. Mitotic IC50 for both drugs was approximately 2 nmol/L.
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Both drugs significantly suppressed microtubule dynamic instability parameters at concentrations ≥2 nmol/L (Table 1, Figs. 3A and B, 4, see also Supplementary Videos S2 and S3). Microtubules grew and shortened more slowly than in control cells, and suppression increased as the concentration of either drug was increased from 1 nmol/L (0.5 × IC_{50}) to 6 nmol/L (3 × IC_{50}; Fig. 4).

Importantly, cabazitaxel (squares) inhibited the growth (Fig. 4A and C) and shortening (Fig. 4B and D) parameters significantly more potently than docetaxel (diamonds). For example, at 2 nmol/L, cabazitaxel and docetaxel suppressed the mean microtubule growth rate by 33% and 19%, respectively, and the mean shortening rate by 59% and 49%, respectively (Fig. 4A and B). The differences in suppression of growth and shortening rates and lengths were not significant at 1 nmol/L of either drug, but were significantly different (between P < 0.05 and P < 0.001) at higher drug concentrations (2–6 nmol/L). Cabazitaxel significantly more potently suppressed the catastrophe frequency than docetaxel at all concentrations tested (P < 0.001; Fig. 4E). At 6 nmol/L, cabazitaxel significantly enhanced rescue frequency as compared with 6 nmol/L docetaxel (P < 0.05; Fig. 4F and Table 1). Overall dynamism was suppressed significantly more strongly (P < 0.01 and P < 0.001) by cabazitaxel than by docetaxel at all concentrations tested (Fig. 4G, P < 0.001). Strikingly, 2 nmol/L cabazitaxel caused the microtubules to pause significantly more (a 136% increase in attenuation, compared with only 38% increase for 2 nmol/L docetaxel (Table 1 and Fig. 4H, P < 0.001).

<p>| Table 1. Effects of cabazitaxel and docetaxel on microtubule dynamic instability in living MCF7 cells at concentrations that arrest mitosis |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control 0</th>
<th>Cabazitaxel, nmol/L (24 hours)</th>
<th>Docetaxel, nmol/L (24 hours)</th>
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</thead>
<tbody>
<tr>
<td>Growth rate (μm/min)</td>
<td>8.1 ± 0.4</td>
<td>5.4 ± 0.2(^a)</td>
<td>6.6 ± 0.3(^a)</td>
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<tr>
<td>Shortening rate (μm/min)</td>
<td>16.2 ± 1.1</td>
<td>6.7 ± 0.3(^a)</td>
<td>8.2 ± 0.7(^a)</td>
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<tr>
<td>Growth length (μm)</td>
<td>2.0 ± 0.2</td>
<td>0.58 ± 0.03(^a)</td>
<td>0.78 ± 0.1(^a)</td>
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<tr>
<td>Shortening length (μm)</td>
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<td>0.55 ± 0.04(^a)</td>
<td>0.70 ± 0.1(^a)</td>
</tr>
<tr>
<td>Attenuation duration (min)</td>
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<td>0.33 ± 0.04(^c)</td>
<td>0.20 ± 0.03(^b)</td>
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<tr>
<td>Time growing (%)</td>
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<td>13</td>
<td>37</td>
</tr>
<tr>
<td>Time shortening (%)</td>
<td>20</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Time attenuated (%)</td>
<td>26</td>
<td>77</td>
<td>38</td>
</tr>
<tr>
<td>Catastrophe frequency (#/min)</td>
<td>2.4 ± 0.1</td>
<td>1.5 ± 0.1(^a)</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>Rescue frequency (#/min)</td>
<td>9.1 ± 0.4</td>
<td>12.6 ± 0.4(^a)</td>
<td>11.6 ± 0.6(^b)</td>
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<tr>
<td>Dynamism (μm/min)</td>
<td>8.9 ± 0.7</td>
<td>1.5 ± 0.2(^c)</td>
<td>3.2 ± 0.5(^c)</td>
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</table>

NOTE: Results are mean values ± SEM, and the corresponding percentage of change from control. Values were analyzed for statistical significance and differ significantly from controls at: \(^a\)P < 0.01, \(^b\)P < 0.05, and \(^c\)P < 0.001 by the Student t test. Dynamicity per microtubule is the length grown and shortened divided by the total life span of the microtubule. At least 25 microtubules were analyzed for each condition. Values for percentage of time growing, shortening, and in the attenuated state were calculated for the entire population of microtubules and, thus, the confidence intervals for these parameters were not calculated.

Both drugs significantly suppressed microtubule dynamic instability parameters at concentrations ≥2 nmol/L (Table 1, Figs. 3A and B, 4, see also Supplementary Videos S2 and S3). Microtubules grew and shortened more slowly than in control cells, and suppression increased as the concentration of either drug was increased from 1 nmol/L (0.5 × IC_{50}) to 6 nmol/L (3 × IC_{50}; Fig. 4).

10 × IC_{50} (10–100 nmol/L) both drugs induced predominantly ball-shaped spindles (Fig. 2K and L).

Both drugs suppress dynamic instability of microtubules in living MCF7 cells at concentrations that arrest mitosis

The effects of cabazitaxel and docetaxel on dynamic instability at plus ends of individual microtubules in the thin peripheral regions of living interphase MCF7 cells were examined after 24 hours of drug incubation with 1, 2, and 6 nmol/L (concentrations that induced increasing degrees of mitotic arrest; Fig. 1D). Figure 3A shows representative frames from time-lapse series displaying length changes of individual microtubules over time in a control cell (top row) and cells incubated for 24 hours with 2 nmol/L cabazitaxel (middle row) and 2 nmol/L docetaxel (bottom row). Length changes were measured, and "life history plots" (Fig. 3B) of changes in length over time were used to determine dynamic instability parameters (Table 1). In control cells, most microtubules were dynamic, undergoing frequent transitions between relatively slow growth and rapid shortening (Supplementary Video S1). Plus ends of microtubules grew at 8.1 ± 0.4 μm/min and shortened at 16.2 ± 1.1 μm/min (Table 1). They underwent catastrophes (transitions to shortening) at a frequency of 2.4 ± 0.12/μmicrotubule/min and rescues (transitions to growth or attenuation from shortening) at a frequency of 9.1 ± 0.4/μmicrotubule/min. Microtubules in controls spent 54% of time growing, 20% of time shortening, and 26% of time in an attenuated or paused state. Their overall dynamism (the total rate of exchange of tubulin at microtubule ends expressed as length per time) was 8.9 ± 0.7 μm/min (Table 1).

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Figure 4. Concentration-dependent effects of cabazitaxel (■) or docetaxel (○) on dynamic instability parameters of microtubules in MCF7 cells at 24 hours compared with controls. A, growth rates; B, shortening rates; C, growing lengths; D, shortening lengths; E, catastrophe frequency; F, rescue frequency; G, dynamicity; and H, attenuation (pause) duration. Results are the mean ± SEM of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by the Student t test. ns, not significant.
additional 30 hours. Washing reduced the amount of drug loss after the washing (20 hours, arrow), vials were rinsed twice during drug incubation for scintillation counting. To determine the rate of drug loss after washing the cells (Fig. 5, arrow). Drug-concentration in the supernatant media was removed at 20 hours, followed by 2 washes and incubation of cells in drug-free medium for an additional 30 hours. *P < 0.02 by the Student t test. ns, not significant.

Cellular uptake of [14C]-cabazitaxel and [14C]-docetaxel

The uptake of 50 nmol/L [14C]-cabazitaxel (–□–) and 50 nmol/L [14C]-docetaxel (–○–) in MCF7 cells over time was determined as described (36). Docetaxel was taken up relatively slowly, attaining an intracellular concentration of 25 μmol/L after 10 hours, and remaining at approximately that level for the next 20 hours (Fig. 5, –○–). Cabazitaxel was taken up significantly more rapidly than docetaxel, attaining an intracellular concentration of 25 μmol/L after only 1 hour, and was retained in cells for as long as 20 hours (Fig. 5, –□–). Cabazitaxel’s uptake was 10 times faster than docetaxel’s, but its accumulation extent was similar to and only slightly greater than docetaxel’s (540- and 500-fold, respectively).

We determined the retention of cabazitaxel and docetaxel after washing the cells (Fig. 5, arrow). Drug-containing media was removed at 20 hours, followed by 2 washes and incubation of cells in drug-free medium for an additional 30 hours. Washing reduced the amount of intracellular cabazitaxel by 15%, from 27 to 23 μmol/L, which was not statistically significant. In contrast, intracellular docetaxel was significantly reduced by 48% (P < 0.02, Student t test), from 25 to 13 μmol/L (Fig. 5).

Discussion

To elucidate the mechanism of action of cabazitaxel, we compared the actions of cabazitaxel and docetaxel in MCF7 cells and found that, in most respects, cabazitaxel was as potent as or more potent than docetaxel. At early incubation times (24 hours), most effects of cabazitaxel were similar to those of docetaxel. The 2 drugs similarly affected interphase and mitotic morphology (Fig. 2), and their effects on mitotic arrest were similar at most concentra-

ions, except for a slight but significantly greater mitotic arrest by cabazitaxel at 24 hours at low drug concentrations (0.3 and 1 nmol/L, P < 0.001; Fig. 1D). In addition, the effects of the 2 drugs on G2–M cell-cycle arrest, apoptosis, and cell death were similar at 24 hours (Fig. 1C, E and F); both drugs induced approximately 85% arrest in G2–M at 24 hours (Fig. 1E and F) but few cells (<3%) entered apoptosis or died (Fig. 1D). The most striking difference between the drugs at 24 hours incubation was that cabazitaxel suppressed all parameters of microtubule dynamic instability significantly more strongly than docetaxel (Fig. 4). We note that a 24-hour drug incubation is not optimum for discerning cell-cycle–dependent differences between the drugs because the doubling time of the EGFP-α-tubulin MCF7 cells we used is 35 hours, and many cells would not have transited a complete cell cycle.

At 48 to 72 hours, all cabazitaxel’s effects were more potent than those of docetaxel. Cabazitaxel inhibited cell proliferation more strongly at 72 hours (Fig. 1B; IC50’s, 0.4 ± 0.1 nmol/L cabazitaxel and 2.5 ± 0.5 nmol/L docetaxel). G2–M cell-cycle arrest by 10 to 100 nmol/L cabazitaxel was maintained at the 24-hour level of 85%, whereas at the same docetaxel concentrations, G2–M arrest decreased and only 55% to 60% of cells were in G2–M (Fig. 1E and F). In addition, at 48 to 72 hours, cabazitaxel induced greater apoptosis/cell death than docetaxel (Fig. 1C). For example, at 100 nmol/L prolonged G2–M arrest induced by cabazitaxel resulted in 37% apoptotic/dead cells, compared with only 26% at 100 nmol/L docetaxel (Fig. 1C).

The reduction in G2–M arrest and apoptosis by docetaxel observed at 48 to 72 hours (Fig. 1E) may result from downstream effects of its less potent suppression of microtubule dynamic instability at 24 hours (Fig. 4) and/or from a possible reduction in intracellular docetaxel concentration at 48 and 72 hours. It is conceivable that long-term incubation with docetaxel induces an increase in expression of multidrug resistance proteins, which in turn might lead to loss of drug from the cells, even in the absence of any washout. Either or both of these phenomena (docetaxel’s lesser suppression of dynamic instability (Fig. 4) and a possible reduction in intracellular docetaxel levels as a result of multidrug resistance protein expression) may contribute to the reduced effects of docetaxel on G2–M arrest and apoptosis that were observed at 48 and 72 hours as well as for the 6-fold difference in inhibition of proliferation measured at 72 hours (Fig. 1B).

Cabazitaxel suppresses microtubule dynamic instability more potently than docetaxel

Both drugs suppressed all parameters of microtubule dynamic instability at low concentrations (1–6 nmol/L, 24-hour incubation) in concentration-dependent manners, leading to highly dynamically stabilized microtubules. Cabazitaxel’s suppression of the growth rate, the catastrophe frequency and dynamicity, and its enhancement of attenuation duration were all significantly greater than docetaxel’s at the >99.9% level of significance (P < 0.001). For example, 2 nmol/L cabazitaxel suppressed the...
dynamicity by 83% in contrast with only 64% suppression by 2 nmol/L docetaxel (Figs. 3 and 4). Statistically significant differences (P < 0.01 or P < 0.05) were also observed between the 2 drugs in suppression of the growth and shortening rates and shortening lengths, with cabazitaxel again having the greatest effects. The least significant difference between the 2 drugs was on enhancement of the rescue frequency (a stabilizing effect) where the only difference was at P < 0.05, at a single concentration, but again cabazitaxel was a significantly more potent stabilizer than docetaxel (Table 1 and Fig. 4).

Taxanes bind along the lengths of microtubules (38), and our preliminary data indicate that cabazitaxel binds with significantly higher affinity to reassembled bovine brain microtubules than does docetaxel (L. Wilson, H. Miller, O. Azarenko, M.A. Jordan; unpublished data). Thus, it is not surprising that cabazitaxel suppressed microtubule dynamic instability in cellular microtubules to a greater extent than docetaxel.

**Differences in uptake and retention of cabazitaxel and docetaxel in MCF7 cells**

Importantly, the rate of uptake of 50 nmol/L cabazitaxel into cells was significantly (10 times) faster than that of 50 nmol/L docetaxel, attaining a plateau concentration of 25 μmol/L at 1 hour and a maximum concentration of 27 μmol/L at 2 hours, whereas docetaxel’s attainment of essentially the same maximum concentration (25 μmol/L) required 10 hours (Fig. 5). Cabazitaxel crosses the lipid bilayer of cellular membranes faster than docetaxel according to the drugs’ calculated lipophilicity values: log P<sub>CAB</sub> (octanol/water) = 3.3 (CAS #183133-96-2) compared with log P<sub>DOC</sub> (octanol/water) = 2.45 (CAS #114977-28-5), which is in agreement with our uptake observations (39–41).

After cell washing at 20 hours, the intracellular concentration of docetaxel was significantly reduced (by 48%) whereas the intracellular levels of cabazitaxel did not change significantly (Fig. 5, arrow). The significantly better retention of cabazitaxel in MCF7 cells is consistent with the much longer terminal γ-half-life reported for cabazitaxel elimination in humans (95 hours vs. 12 hours for docetaxel; refs. 15 and 16). As mentioned above, cabazitaxel seems to bind with higher affinity to microtubules than docetaxel. It is also conceivable that docetaxel treatment might induce increased expression of multidrug resistance proteins that could promote drug efflux.

**Did the differential rate of uptake of cabazitaxel and docetaxel and their different susceptibilities to loss upon washout affect the measured values of suppression of dynamic instability?**

We chose to examine effects on microtubule dynamic instability after 24 hours drug incubation specifically in order to examine the cells after plateau intracellular drug concentrations were attained, as evidenced in the drug uptake experiments (Fig. 5). However, rates of drug uptake and loss vary with the concentration of drug added to cells (42). One can ask if docetaxel might have been more rapidly pumped out of cells than cabazitaxel before the dynamic instability experiments, in particular if the intracellular drug levels were different in the dynamic instability experiments as compared with the uptake experiments. To examine this question, we calculated and compared the approximate intracellular drug concentrations in the uptake and dynamic instability experiments. We know from past experiments that cells take up all or nearly all of a taxane available to them in culture medium (M.A. Jordan; unpublished data). Thus, we compared the ratio of cell number to total drug in the medium to determine whether the intracellular drug concentrations were of the same magnitude in the 2 kinds of experiments. The numbers of cells per volume ranged between a low of 1.5 × 10<sup>4</sup> cells/mL for dynamic instability experiments to a high of 5 × 10<sup>4</sup> cells/mL for drug uptake experiments. The drug concentrations in dynamic instability determinations ranged from 1 to 6 nmol/L. The drug concentration used in uptake experiments was 50 nmol/L. Therefore, the ratio of available drug to cell number in the dynamic instability measurements was between (1 nmol/L)/(1.5 × 10<sup>4</sup> cells/mL) and (6 nmol/L)/(1.5 × 10<sup>0</sup> cells/mL). In uptake experiments, the ratio was (50 nmol/L)/(5 × 10<sup>4</sup> cells/mL). Arithmetically simplifying the above ratios, the drug concentrations on a per cell basis were 0.66 × 10<sup>-16</sup> and 4 × 10<sup>-16</sup> moles per cell for dynamic instability measurements as compared with 10 × 10<sup>-16</sup> moles per cell for the uptake experiments. Thus, the intracellular drug concentrations in the dynamic instability experiments were of the same order of magnitude as those in the uptake experiments in which the plateau concentrations stayed constant for at least 14 hours, suggesting that our comparison of intracellular effects on dynamic instability parameters is likely valid and that drug concentrations were maintained over time in the dynamic instability experiments as well as they were clearly maintained in the uptake/retention experiments.

Ultimately, it will be important to compare the effects of the 2 drugs on dynamic instability of purified microtubules to be absolutely sure that cabazitaxel suppresses microtubule dynamic instability more strongly than docetaxel. These experiments are underway. From the above calculations, we predict that the differences between the effects of the 2 drugs on dynamic instability observed in cells (Fig. 4) will hold true with purified microtubules. Therefore, we suggest that the significantly greater inhibitory effects of cabazitaxel on microtubule dynamic instability at 24 hours are responsible for the significantly greater potency of cabazitaxel at 48 to 72 hours. The greater potency of cabazitaxel may be further enhanced by its greater retention in cells as compared with that of docetaxel.

Overall, the results indicate that, although cabazitaxel is structurally very similar to docetaxel and most likely binds to microtubules at the same and/or an overlapping
binding site, it suppresses microtubule dynamic instability significantly more strongly than docetaxel and is taken up more quickly and can be retained longer in cells, thus producing a longer-lasting G₂-M arrest followed by increased apoptosis. These differences likely play a major role in cabazitaxel’s enhanced clinical activity over docetaxel regimens.

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M.A. Jordan and L. Wilson report receiving commercial research grants from Sanofi. No potential conflicts of interest were disclosed by the other authors.

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