Class III β-Tubulin Isotype Predicts Response in Advanced Breast Cancer Patients Randomly Treated Either with Single-Agent Doxorubicin or Docetaxel

Carlos M. Galmarini,1 Isabelle Treilleux,2 Fatima Cardoso,3 Chantal Bernard-Marty,3 Virginie Durbecq,3 David Gancberg,4 Marie-Christine Bissery,5 Marianne Paesmans,3 Denis Larsimont,3 Martine J. Piccart,3 Angelo Di Leo,6 and Charles Dumontet1

Abstract

Purpose: To evaluate the role of microtubule-associated variables as potential predictors of response and clinical outcome in patients with advanced breast cancer receiving single-agent docetaxel or doxorubicin chemotherapy.

Experimental Design: The analysis was done on 173 tumor samples from patients with locally advanced or metastatic breast cancer who have participated in the TAX-303 phase III trial in which patients were randomly assigned to receive docetaxel or doxorubicin. Expression of total α- and β-tubulin, classes II to IV β-tubulin isotypes, and τ protein was evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded tumors from the primary breast cancer.

Results: We observed that patients with “high” expression of class III β-tubulin isotype had a higher probability of response to docetaxel than to doxorubicin treatment (odds ratio, 1.9; 95% confidence interval, 1.01-3.7; P = 0.05). No difference was observed in terms of time to progression or in terms of overall survival.

Conclusions: This study suggests that the superiority of docetaxel over doxorubicin seems to be confined to the subgroup of patients with “high” expression of class III β-tubulin isotype.

Numerous compounds and chemotherapy regimens have proven efficacious in patients with advanced breast cancer (1). Doxorubicin single-agent therapy has long been considered as the standard chemotherapeutic treatment at this stage of disease, with response rates (RR) of 25% to 33% (2). However, doxorubicin administration is associated with serious toxic side effects (myelosuppression, cumulative dose-dependent cardiotoxicity, and risk of secondary leukemia) that limit its use in the palliative setting (3–5). Another common agent currently used in treating advanced breast cancer patients is docetaxel (6). This agent binds to β-tubulin, a major protein in mitotic spindles, and halts cell division in metaphase. When administered as single-agent therapy, docetaxel treatment yielded RR of 30% to 60% in patients with no prior therapy and 20% to 45% in patients who had received prior chemotherapy (7, 8). The side-effect profile of docetaxel is quite different from that of doxorubicin (myelosuppression, neuropathy, fluid retention, and nail disorders; refs. 7, 8). Although docetaxel has shown, on average, higher RR than doxorubicin in this clinical subset, it is still not clear which individual patient might benefit more from docetaxel or from doxorubicin chemotherapy.

In recent years, efforts have been made to find molecular markers capable of predicting the efficacy of taxanes that would lead to a rationalized and biologically driven treatment approach. Several studies searching for biomarkers that would predict a clinical response to taxanes in breast cancer have been published but available data are largely contradictory. For example, retrospective studies have suggested that overexpression of HER-2 was associated with enhanced taxane efficacy (9, 10). However, other studies seem to suggest no interactions between HER2 status and taxane therapy (11, 12). Similar conflicting results were observed for other biomarkers such as the apoptotic index (9), p53 (12, 13), proliferation markers (14), and angiogenic-related markers (15, 16). Thus, markers that help predict whether a patient might benefit more from a taxane-based than from an anthracycline-based regimen are clearly needed.

Based on preclinical data, microtubule-associated variables are attractive biomarker candidates, especially because they can be easily evaluated by immunohistochemistry on archival tumor samples. Numerous reports have shown that the expression of total α- and/or β-tubulin and individual β-tubulin isotypes as well as alterations of the expression in microtubule-associated proteins (MAP4, STOP, τ) are correlated in vitro.
An extensive description of the TAX-303 trial reported that with the sensitivity to taxanes (17–19). Rouzier et al. have associated variables (total h-tubulin, classes II to IV β-tubulin isotypes, and τ protein expression) as potential predictors of response to single-agent docetaxel chemotherapy and clinical outcome in patients with advanced breast cancer. Patients included in this retrospective study participated in a phase III clinical trial (TAX-303) in which docetaxel or doxorubicin was randomly administered for advanced disease (24). We thus decided to evaluate the role of microtubule-associated variables (total α- and β-tubulin, classes II to IV β-tubulin isotypes, and τ protein expression) as potential predictors of response to single-agent docetaxel chemotherapy and clinical outcome in patients with advanced breast cancer. Patients included in this retrospective study participated in a phase III clinical trial (TAX-303) in which docetaxel or doxorubicin was randomly administered for advanced disease (24). Because of its unique design, this trial provided an ideal opportunity to investigate the predictive value of molecular markers in an attempt to identify a specific subgroup of patients deriving the highest benefit from the use of docetaxel.

### Materials and Methods

**Patients.** The analysis was done on tumor samples from patients with locally advanced or metastatic breast cancer who have participated in the TAX-303 trial. This was a randomized, multicenter, nonblinded, prospective, phase III study in which patients were assigned to receive an i.v. infusion of docetaxel (100 mg/m² every 3 weeks) or doxorubicin (75 mg/m² every 3 weeks). An extensive description of the TAX-303 trial has already been published (24). Briefly, eligibility criteria included histologically or cytologically proven metastatic breast cancer, ages 18 to 75 years, assessable disease, performance status of at least 60 (Karnofsky index), and no previous treatment with chemotherapy other than cyclophosphamide, methotrexate, and fluorouracil either in the adjuvant or in the metastatic setting. Before the start of the trial, ethics committee approval and informed patient consent were obtained. A complete tumor assessment was done before registration in the study, during treatment, and at least every month until disease progression during the follow-up period (24). Objective responses were evaluated according to the WHO criteria (25).

**Sample collection.** The Leon Berard Cancer Center was provided with one paraffin-embedded sample representative of the primary tumor for each patient entered in the clinical trial and for whom sufficient and accessible paraffin-embedded tumor material was still available. Immunohistochemical analyses were thus done in available material from 174 of the 326 patients entered in the clinical trial (53%).

**Immunohistochemical analyses.** Expression of total α- and β-tubulin, classes II to IV β-tubulin isotypes, and τ protein was evaluated by immunohistochemistry. The primary antibodies used for immunohistochemistry were α-tubulin (1:600; clone DM1A: Sigma); β-tubulin (1:3,000; clone TUB2; Sigma); class II β-tubulin (1:700; clone 7H9; provided by Anthony Frankfurter); class III β-tubulin (1:100; clone TUJ1; provided by Anthony Frankfurter); class IV β-tubulin (1:500; Sigma); and τ (1:200; Cedarlane Laboratories).

To evaluate total α- and β-tubulin and classes II and III β-tubulin isotype expression, 4 μm sections were deparaffinized in xylene, rehydrated, and submitted to heat-induced antigen retrieval for 15 min in 10 mmol/L citrate buffer (pH 7). After cooling at room temperature for 20 min, tissue sections were rinsed in PBS and treated with 5% H₂O₂ for 15 min to block endogenous peroxidase. After washing with PBS, the slides were incubated with the primary antibody for 60 min at room temperature. After washing with PBS, tissue sections were incubated at room temperature for 30 min with a biotinylated goat anti-rabbit or mouse secondary antibody and washed with PBS. The avidin-biotin peroxidase complex was then added for 30 min (StreptABComplex/HRP Duet; DAKO). After washing with PBS, the slides were stained with Harris hematoxylin and submitted for interpretation.

To evaluate class IV β-tubulin isotype and τ protein detection, slides were treated with xylene to remove paraffin and ethanol (100% to 70%) and obtain rehydration with 5-min baths. Epitopes were restored in a 10 mmol/L citrate buffer (pH 6) by submitting to heat for 15 min in a microwave oven. Exposure to 3% H₂O₂ for 30 min was used to inhibit endogenous peroxidases. PBS with 1% FCS was used for both blocking and dilution of antibody solutions. Primary antibodies were left on tissues overnight at 4°C in a moist room. Tissue sections were then incubated with biotinylated secondary antibodies for 40 min at room temperature. Signal amplification was provided by a streptavidin

### Table 1. Patient characteristics at diagnosis in the study group and in the TAX-303 clinical trial group

<table>
<thead>
<tr>
<th></th>
<th>Immunohistochemistry study</th>
<th>Total clinical trial population</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total Docetaxel group</td>
<td>Doxorubicin group</td>
</tr>
<tr>
<td>No. patients</td>
<td>174</td>
<td>86</td>
</tr>
<tr>
<td>Age (median)</td>
<td>52</td>
<td>53</td>
</tr>
<tr>
<td>Karnofsky performance</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Status</td>
<td>1,00</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2,00</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>&gt;2</td>
<td>36</td>
</tr>
<tr>
<td>Metastatic sites</td>
<td>Soft tissue only</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Viscera</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Adjuvant chemotherapy only</td>
<td>93</td>
</tr>
<tr>
<td>Outcome</td>
<td>Overall RR (%)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Complete RR (%)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>TTP (mo)</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>OS (mo)</td>
<td>14.4</td>
</tr>
</tbody>
</table>

*Significant difference between the treatment arms in the clinical trial group (P = 0.005) as reported by Chan et al (24).
peroxidase complex (DAKO). The signal was detected by a colorimetric method using aminoethyl-carbazole. All of the slides were examined and scored independently by a pathologist (I.T.) and a technician without knowledge of the patient data. Interrater reproducibility was 97% and discordant cases were reviewed by both scorers. A minimum of 200 cells were counted. Only cytoplasmic staining was taken into account. Possible scores ranged from 0 to 100%.

**Statistical methods.** The levels of expression of immunohistochemical variables in the two treatment groups were compared by applying a Mann-Whitney U test to these values considered as continuous variables. Cutoffs for definition of “low” or “high” expression were defined based on the median values observed in the patient population. These values were then used as dichotomic variables for correlating biological and clinical variables. A patient was defined as having a “high expression” (“low expression”) of a given biological marker if his tumor had a percentage of positive cells greater (lower) than the median value observed in the patient population. Clinical covariates that were expected to play an important prognostic role in advanced breast cancer were also studied and considered as dichotomic values. These included Karnofsky performance status (poor: performance status < 80, good: performance status ≥ 80), visceral involvement (present; absent), and number of metastatic sites (<3, ≥3). Comparisons between categorical variables were based on the $\chi^2$ test.

Time to progression (TTP) was calculated from the date of randomization until progression or death. Patients who received any further antitumor treatment before disease progression were censored at the date of the last tumor assessment before the start date of the new antitumor treatment. Overall survival (OS) was calculated from the date of randomization to the date of death for any reason or to the date of last follow-up (censored event). TTP and OS curves were calculated.

### Table 2. Expression of microtubule-associated variables in tissue samples

<table>
<thead>
<tr>
<th></th>
<th>Total group (range)</th>
<th>Docetaxel group (range)</th>
<th>Doxorubicin group (range)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total α-tubulin</td>
<td>90 (40-90)</td>
<td>90 (40-90)</td>
<td>90 (50-90)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total β-tubulin</td>
<td>90 (0-90)</td>
<td>85 (0-90)</td>
<td>90 (20-90)</td>
<td>0.16</td>
</tr>
<tr>
<td>Class II β-tubulin</td>
<td>50 (0-90)</td>
<td>50 (0-90)</td>
<td>60 (0-90)</td>
<td>0.50</td>
</tr>
<tr>
<td>Class III β-tubulin</td>
<td>50 (0-90)</td>
<td>50 (0-90)</td>
<td>40 (0-90)</td>
<td>0.43</td>
</tr>
<tr>
<td>Class IV β-tubulin</td>
<td>80 (0-90)</td>
<td>80 (10-90)</td>
<td>80 (0-90)</td>
<td>0.68</td>
</tr>
<tr>
<td>ε-tubulin</td>
<td>70 (0-90)</td>
<td>70 (0-90)</td>
<td>70 (0-90)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**NOTE:** Values in table are median of positive cells in each sample.

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**Fig. 1.** A, B, immunostaining of anti-class III β-tubulin in nonneoplastic mammary tissue. C, D, invasive carcinoma of the mammary gland strongly stained with anti-class III β-tubulin antibody. Ninety percent of tumor cells are positive for class III β-tubulin.
Table 3. Correlation between microtubule-associated variables and RR

<table>
<thead>
<tr>
<th>% RR (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Doctaxel group</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 52.8 (35.6-70)</td>
<td>48.3 (29.0-67.6)</td>
</tr>
<tr>
<td>&gt;Median 38.5 (22.5-54.5)</td>
<td>22.0 (10.1-33.9)</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 42.9 (25.6-60.1)</td>
<td>37.5 (21.8-53.2)</td>
</tr>
<tr>
<td>&gt;Median 46.3 (30.4-62.3)</td>
<td>25.0 (11.0-39.0)</td>
</tr>
<tr>
<td>Class IV</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 50.0 (26.0-74.0)</td>
<td>36.4 (14.5-58.2)</td>
</tr>
<tr>
<td>&gt;Median 44.6 (31.2-58.1)</td>
<td>29.8 (15.6-42.1)</td>
</tr>
<tr>
<td>T</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 46.7 (27.7-65.6)</td>
<td>32.0 (12.3-51.7)</td>
</tr>
<tr>
<td>&gt;Median 44.7 (30.0-59.4)</td>
<td>31.5 (18.7-44.3)</td>
</tr>
<tr>
<td>α</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 38.7 (20.5-56.9)</td>
<td>25.7 (10.5-40.9)</td>
</tr>
<tr>
<td>&gt;Median 50.0 (34.6-65.4)</td>
<td>35.6 (21.0-50.0)</td>
</tr>
<tr>
<td>β</td>
<td></td>
</tr>
<tr>
<td>&lt;Median 43.2 (26.5-60.0)</td>
<td>33.3 (17.2-49.5)</td>
</tr>
<tr>
<td>&gt;Median 47.4 (30.7-64.0)</td>
<td>31.8 (17.5-46.1)</td>
</tr>
</tbody>
</table>

given according to the Kaplan-Meier method and compared by the log-rank test. Differences were considered significant if \( P \leq 0.05 \) (two sided). All statistical analyses were done using Statistica 7.0.

Results

Patient characteristics and treatment administration. Table 1 summarizes the main characteristics of patients included in this study as well those included in the clinical phase III trial. The most important negative prognostic factors (poor performance status, presence of visceral metastasis, and involvement of three or more sites) were well represented and balanced between the two treatment arms in our groups. There were no significant differences between the immunohistochemistry group and the clinical trial group.

Of the total population, 66 patients responded to treatment yielding an overall RR of 38%, with a complete RR of 6%. When comparing treatment arms, the overall RR was higher with docetaxel than with doxorubicin (42% versus 34%, respectively; Table 1); however, the difference between treatment groups did not reach statistical significance in this subgroup of patients (odds ratio, 1.4; 95% confidence interval, 0.7-2.6; \( P = 0.1 \)), whereas it was statistically significant in the subgroup of patients with greater than median class III \( \beta \)-tubulin (46.3 versus 25%; \( P = 0.05 \); odds ratio, 1.9; 95% confidence interval, 1.01-3.7; \( P = 0.05 \)). In contrast, in patients with “low” expression of class III \( \beta \)-tubulin isotype, no significant difference in the RR to docetaxel and doxorubicin was observed. Similarly, no statistically significant difference in the RR to docetaxel and doxorubicin was observed in the cohorts defined by other microtubule-associated variables. An interaction analysis was done by performing a regression analysis taking into account the treatment arm, the level of expression of class III \( \beta \)-tubulin, and an interaction covariate. No interaction was observed, but this may be due to the low power of interaction tests on small samples.

In univariate analysis, microtubule-associated variables and clinical covariates were not correlated to clinical outcome (TTP and OS) in the entire group or when considering patients receiving docetaxel or doxorubicin treatment (data not shown). It should, however, be emphasized that, in the clinical trial report on 326 patients, the significant difference in RR between the treatment arms was not associated with a significant difference in TTP or OS, although there was a trend toward longer TTP in the docetaxel group.

Discussion

In the present study, we show that advanced breast cancer patients with “high” expression of class III \( \beta \)-tubulin isotype had a higher probability of response to docetaxel than to doxorubicin treatment. In this subset of patients, the odds for response to docetaxel in patients receiving docetaxel were 1.9 times higher than in patients receiving doxorubicin. In this patient population, we did not observe a difference in terms of TTP or OS in these subgroups. This negative result may be due to the limited number of patient samples available for this study, because in the clinical trial, which accrued 326 patients, the difference in RR between patients receiving docetaxel or doxorubicin was not associated with a significant difference in survival.

Microtubules are polymers consisting of tubulin dimers containing one \( \alpha \)-tubulin and one \( \beta \)-tubulin molecule and a variety of microtubule-associated proteins (26). In humans, tumor samples with a median positivity of 90%. The expression of \( \beta \)-tubulin isotypes differed significantly with a median positivity of 50% for classes II and III \( \beta \)-tubulin isotype and 80% for class IV \( \beta \)-tubulin isotype. \( \tau \) protein was also expressed in the majority of the samples (70%). Examples of samples with negative expression and positive expression for class III \( \beta \)-tubulin isotype are shown in Fig. 1. No significant difference in microtubule-associated variable expression was observed between the two treatment groups. There were no significant correlations between the level of expression of the different microtubule-associated variables, except between tubulin II and \( \tau \) protein (see Supplementary Table S1).

Microtubule-associated variable expression and patient outcome. We analyzed the correlation between microtubule-associated variables (dichotomized as lower than or greater than median value in the study population) and RR to docetaxel and doxorubicin. The univariate analysis showed a statistically significant correlation between RR and treatment arm in patients with greater than median class III \( \beta \)-tubulin isotype expression (Table 3). In patients with “high” expression of class III \( \beta \)-tubulin isotype, docetaxel was more active than doxorubicin (46.3 versus 25%; \( P = 0.05 \); odds ratio, 1.9; 95% confidence interval, 1.01-3.7; \( P = 0.05 \)). In contrast, patients with “low” expression of class III \( \beta \)-tubulin isotype, no significant difference in the RR to docetaxel and doxorubicin was observed. Similarly, no statistically significant difference in the RR to docetaxel and doxorubicin was observed in the cohorts defined by other microtubule-associated variables. An interaction analysis was done by performing a regression analysis taking into account the treatment arm, the level of class III \( \beta \)-tubulin isotype expression, and an interaction covariate. No interaction was observed, but this may be due to the low power of interaction tests on small samples.

In univariate analysis, microtubule-associated variables and clinical covariates were not correlated to clinical outcome (TTP and OS) in the entire group or when considering patients receiving docetaxel or doxorubicin treatment (data not shown). It should, however, be emphasized that, in the clinical trial report on 326 patients, the significant difference in RR between the treatment arms was not associated with a significant difference in TTP or OS, although there was a trend toward longer TTP in the docetaxel group.
there are six β-tubulin protein isotypes classified as classes I, II, III, IVa, IVb, and VI (27). Dozier et al. recently showed a similar broad distribution of β-tubulin isotypes in normal and tumor breast tissues with classes I, II, and IV being the most abundantly expressed isotypes (21). Microtubule-associated proteins constitute a complex family of proteins that includes MAP2, MAP4, Mip-90, τ, and STOP (28, 29). Thus, every microtubule contains different isotypes in various proportions and is likely to bind a large variety of microtubule-associated proteins. Differences among the isotype composition in combination with interactions with microtubule-associated proteins can greatly influence microtubule dynamics (30–32). Because taxanes induce cytotoxicity by inhibiting microtubule dynamics, it is possible that alterations in β-tubulin isotype content and microtubule-associated proteins may alter sensitivity to taxanes.

Only a few previous studies have analyzed the importance of the different β-tubulin isoform expression in response and clinical outcome in breast cancer patients. A preliminary study on 41 patients published by ourselves (22) evaluated the expression of α- and β-tubulin, classes II to IV β-tubulin isotypes, and τ expression by immunohistochemistry. In this report, overexpression of class II β-tubulin isotype at the protein level was predictive of a worse response to docetaxel therapy. No correlation was found between response to docetaxel and the level of expression of the other microtubule-associated variables. In another study, Paradiso et al. reported that overexpression of class III β-tubulin isotype was predictive of progression after epirubicin/docetaxel treatment in 70 patients with metastatic breast cancer (23). Rouzier et al. analyzed τ protein expression in patients with breast cancer and suggested that low τ values are associated with greater sensitivity to paclitaxel (20).

In other clinical settings, such as non-small cell lung cancer and ovarian cancer, overexpression of class III β-tubulin isotype has also been associated with poor prognosis in patients treated with taxanes (33–36). Similar observations have been made in other types of tumors including ovarian cancer (17) and carcinomas of unknown origin (37). β-Tubulin III has also been found to be expressed in pancreatic adenocarcinoma and glioblastoma (38, 39). Of interest, inhibition of class III tubulin has been shown to sensitize tumor cells to microtubule-binding agents (40).

It is not yet clear by what mechanisms the alteration in microtubule components can alter sensitivity to antitubulin agents. One explanation could be that microtubules contained in the tumor cells have different dynamic properties due to different isotype composition and may thus present differential sensitivity to taxanes (32, 41, 42). In some cell line models, ability to survive paclitaxel exposure is accompanied by alterations in total tubulin content (43) and tubulin isoform composition (17, 44). Although it has proven difficult to show a causal relationship between any of these changes and the response to taxanes, one can readily envision more stable, less dynamic microtubules being more acutely affected by the microtubule-stabilizing properties of docetaxel. A second explanation could also be that taxanes would have a preferential binding to certain isotypes, but evidence in support of this is not available. Finally, the third hypothesis suggests that the critical factor that regulates response to taxanes is the total amount of polymerizable tubulin and not the tubulin isoform level. In accordance with this hypothesis, we and others have shown that the total amount of polymerizable tubulin is decreased in taxane-resistant cells (19, 45).

This explorative study has some clear limitations such as its retrospective design, modest study sample size, heterogeneity of the eligible patients (different number of previous lines of treatment), and no availability of tumor samples in half of the TAX-303 patient population. Nevertheless, this study offers the exclusive advantage of being designed ideally for a head-to-head comparison between docetaxel and doxorubicin (24). In fact, the TAX-303 study is an ideal set to investigate predictive markers because treatment assignment was randomized, therapy was based on a single-agent treatment, and drugs were given at the standard doses.

In conclusion, the present study has investigated the value of microtubule-associated variable expression as biomarkers in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or with single-agent docetaxel. The results of this study suggest that the superiority of docetaxel over doxorubicin seems to be confined to the subgroup of patients with “high” expression of class III β-tubulin isotype, whereas in the subgroup of patients with “low” expression of this isotype doxorubicin appears to be at least as effective as docetaxel. Future prospective studies evaluating the value of this marker in breast cancer patients receiving antitubulin-based regimens and unrelated regimens should be done to determine the clinical usefulness of this marker.

Disclosure of Potential Conflicts of Interest

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