Association of the CYP1B1*3 allele with survival in patients with prostate cancer receiving docetaxel

Tristan M. Sissung,1 Romano Danesi,5 Douglas K. Price,2 Seth M. Steinberg,3 Ronald de Wit,6 Muhammad Zahid,7 Nilesh Gaikwad,7 Ercole Cavalieri,7 William L. Dahut,2 Dan L. Sackett,4 William D. Figg,1,2 and Alex Sparreboom1

1Clinical Pharmacology Program, 2Medical Oncology Branch, and 3Biostatistics and Data Management Section, Center for Cancer Research, National Cancer Institute; 4Laboratory of Integrative and Medical Biophysics, National Institute of Child Health and Human Development, Bethesda, Maryland; 5Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, Pisa, Italy; 6Department of Medical Oncology, Erasmus MC-Daniel den Hoed Cancer Center, Rotterdam, the Netherlands; and 7Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska

Abstract
Using a single nucleotide polymorphism association study in 52 men with prostate cancer receiving docetaxel, we found that individuals carrying two copies of the CYP1B1*3 polymorphic variant had a poor prognosis after docetaxel-based therapies compared with individuals carrying at least one copy of the CYP1B1*1 allele (30.6 versus 12.8 months; P = 0.0004). The association between CYP1B1*3 and response to therapy was not observed in similar subjects receiving non-taxane-based therapy (P = 0.18). The systemic clearance of docetaxel was also unrelated to CYP1B1 genotype status (P = 0.39), indicating that the association of CYP1B1*3 with clinical response is not due to docetaxel metabolism. To explain these results, we hypothesized that an indirect gene-drug interaction was interfering with the primary mechanism of action of docetaxel, tubulin polymerization. We therefore conducted tubulin polymerization experiments with taxanes in the presence or absence of certain CYP1B1 estrogen metabolites, which are known to bind to nucleophilic sites in proteins and DNA, that revealed the primary estrogen metabolite of CYP1B1, 4-hydroxyestradiol (4-OHE2), when oxidized to estradiol-3,4-quinone strongly inhibits tubulin polymerization. The 4-OHE2 is also formed more readily by the protein encoded by the CYP1B1*3 allele, validating further our data in patients. Furthermore, estradiol-3,4-quinone reacted in vitro with docetaxel to form the 4-OHE2-docetaxel adduct. This pilot study provides evidence that CYP1B1*3 may be an important marker for estimating docetaxel efficacy in patients with prostate cancer. This link is likely associated with CYP1B1*3 genotype-dependent estrogen metabolism. [Mol Cancer Ther 2008;7(1):19–26]

Introduction
Docetaxel is one of the most frequently prescribed anticancer agents. It is derived from the taxane family of agents that stabilize microtubules at the mitotic spindle resulting in cell cycle arrest during mitosis followed by apoptosis. Despite the notable success of docetaxel in the treatment of various cancers, including androgen-independent prostate cancer (AIPC), high variability in clinical response to docetaxel has been observed (1). This variability can be attributed in part to a poor understanding of interindividual differences in docetaxel pharmacokinetics and pharmacodynamics. Given the clinical importance of docetaxel, genetic markers with predictive power to assess interindividual differences resulting in clinical outcome before docetaxel administration are urgently needed, and a mechanistic understanding of these gene-drug interactions could be useful in improving the treatment efficacy of docetaxel.

Cellular sensitivity toward docetaxel has been associated previously with the isozyme cytochrome P450 1B1 (CYP1B1) in preclinical studies. CYP1B1 is a heme-thiolate mono-oxygenase that is involved in the mono-oxygenation of a variety of substrates, including steroids and xenobiotics. It is expressed in several tumors, including prostate (2) and breast cancers (3), where it is differentially up-regulated within tumor cells compared with surrounding normal tissue (4, 5). Although CYP1B1 does not metabolize docetaxel (6), CYP1B1 overexpression results in reversible docetaxel resistance (3, 7). Two hypotheses have been proposed to explain the association between CYP1B1 metabolism and clinical outcome. The first states that, by binding intracellular docetaxel, CYP1B1 reduces the effective concentration of drug in cancer cells, thereby influencing drug efficacy. The second states that CYP1B1 metabolism results in increased levels of certain genotoxic compounds, thereby influencing tumor biology (8).
Data from several studies suggest that CYP1B1 catalysis is responsible for the formation of certain estrogen metabolites, including the minor metabolite, 2-hydroxyestradiol (2-OHE2), and the major metabolite, 4-hydroxyestradiol (4-OHE2). 2-OHE2 and 4-OHE2 can then undergo subsequent oxidation to form highly reactive estradiol-2,3-quinone or estradiol-3,4-quinone (E2-3,4-quinone), respectively. The E2-3,4-quinone reacts with the purines in the DNA to form depurinating adducts. The apurinic sites generated in the DNA lead to mutations that could initiate breast, prostate, and other human cancers (9). However, these estrogen metabolites may also react with intracellular proteins, including tubulin (10). Estrogen exposure has been implicated in the etiology of prostate cancer (11). Because CYP1B1 is overexpressed in prostate cancer cells (5), where through the generation of estrogen metabolites that bind tubulin, and potentially also react with docetaxel, CYP1B1 catalysis could interfere with docetaxel-mediated tubulin stabilization and also structurally alter the drug.

The CYP1B1 gene is highly polymorphic, and several nonsynonymous single nucleotide polymorphisms contained within the CYP1B1 gene have been identified that alter the expression and/or activity of the encoded protein. Of these, the CYP1B1*3 (4326C>G; L432V) allele has been characterized by both increased expression and enzyme kinetics of CYP1B1 toward several substrates (12–15). Although it is currently controversial, several studies suggest that the CYP1B1*3 polymorphism encodes a protein that more efficiently metabolizes estrogen into 4-OHE2, thereby increasing the intracellular ratio of 4-OHE2/2-OHE2 (reviewed in ref. 5). Against this background, we hypothesized that the CYP1B1*3 genotype could modulate the therapeutic response to docetaxel treatment by catalyzing the more efficient formation of an estrogen metabolite that both binds to microtubules and reacts with docetaxel, thereby interfering with the mechanism of action of docetaxel.

Materials and Methods

Patients and Treatment

CYP1B1 genotypes were retrospectively evaluated on germ-line DNA obtained from 52 men with AIPC treated on one of the following studies: single-agent docetaxel (1-h infusion; 30 mg/m²; n = 25; ref. 14), docetaxel in combination with estramustine and thalidomide (30-min infusion; 30 mg/m²; n = 20; ref. 16), or docetaxel in combination with prednisone (1-h infusion; 75 mg/m²; n = 7; ref. 17). CYP1B1 genotypes were also assessed in 145 patients with AIPC treated with suramin-based therapy alone (n = 36) or in combination with amino-glutethimide (n = 27) or with flutamide plus leuprolide (n = 41) or with thalidomide alone (n = 41; refs. 18–21). Baseline demographic data for all patients are shown in Table 1. The study protocols from all studies were approved by the institutional review board of their respective institutions, and all patients provided written informed consent.

Pharmacokinetics of Docetaxel

Blood specimens were obtained from a separate cohort of patients receiving docetaxel (1-h infusion; 75 or 85 mg/m²; n = 23) as described previously (22). Evaluation of docetaxel pharmacokinetics was done using samples obtained immediately before drug infusion and at 0.5, 1, 2, 4, 6, and 24 h after start of infusion. Concentrations of docetaxel in plasma were determined by a validated reverse-phase high-performance liquid chromatography method with UV detection (23). Pharmacokinetic variables of docetaxel were obtained by noncompartmental analysis using WinNonlin Professional version 5.0 (Pharsight), model 202 (plasma data, constant infusion).

Genotyping

DNA was extracted from plasma using a QiaBlood DNA extraction kit (Qiagen) and stored at 4°C. Genotyping was conducted via direct sequencing at the CYP1B1*3 locus using the following PCR primers: CYP1B1*3 F1–GGATCCTGATGTCAGACTCG and R1–TGACAGCACTATCAAAGGACT. A 50-µL reaction was prepared for PCR amplification. The reaction consisted of 1× PCR buffer, 2 mmol/L of each of the four deoxynucleotide triphosphates, 1.5 mmol/L magnesium chloride, and 1 unit Platinum Taq DNA polymerase. PCR conditions were as follows: 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 30 s, with a final 7-min cycle at 72°C. Direct nucleotide sequencing PCR was

Table 1. Patient demographics by CYP1B1*3 genotype

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Docetaxel-treated cohort</th>
<th>Suramin/thalidomide-treated cohort</th>
</tr>
</thead>
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<td>All</td>
<td>WT or Het</td>
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<td>Total entered</td>
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<td>2.0 (1.7-2.5)</td>
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<tr>
<td>Gleason score*</td>
<td>8 (1-10)</td>
<td>8 (1-10)</td>
</tr>
</tbody>
</table>

NOTE: Continuous data are given as median with range in parentheses, and categorical data are given as number of patients with percentage of the total population in parentheses. CYP1B1*3 genotype: WT, wild-type; Het, heterozygous variant; Var, homozygous variant.

*Gleason scores for two patients were unavailable.
conducted using the Big Dye Terminator Cycle Sequencing Ready Reaction kit version 1.1 on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The primer sequences for this reaction were as follows: F2-TGCTTCTACATTCCTCAGGCC and R2-GGTGAGCCAGGATGGAGATGA. The products were run on a 3% agarose gel and purified with a QIAquick Gel Extraction Kit (QIAGEN). The purified products were then sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction kit version 1.1 on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The primer sequences for this reaction were as follows: F2-TGCCTGTCACTATTCCTCAGGCC and R2-GGTGAGCCAGGATGGAGATGA.

**In vitro Tubulin Polymerization**

Rat brain tubulin was purified from microtubule protein (24) by differential polymerization as described previously (25). Tubulin polymerization reactions (50 μL) were done in half-area microwell plates, and polymerization was monitored by absorbance at 350 nm. The final concentrations were as follows: tubulin, 10 μmol/L; paclitaxel or docetaxel, 10 μmol/L; GTP, 1 mmol/L; test compound, 50 μmol/L; DMSO 4% [(v/v)]; 0.2% for docetaxel]; all in 0.1 mol/L Pipes, 1 mmol/L MgCl₂ (pH 7.0). Tubulin was preincubated with compounds or solvent controls at 37°C for 15 min followed by cooling on ice for 5 min. GTP and paclitaxel or docetaxel were then added and mixed, and the plate was moved to a SpectraMax plate reader (Molecular Devices), preset to 30°C. Absorbance at 350 nm was recorded with time for 20 min.

**Reaction of E2-3,4-Quinone with Docetaxel**

The synthesis of E2-3,4-quinone from 4-OHE2 was carried out according to published procedures (26). Freshly synthesized E2-3,4-Q (1.7 μmol in acetonitrile) was added to a solution of docetaxel (3.4 μmol) dissolved in 200 μL acetonitrile and 500 μL phosphate buffer and stirred at 37°C. The reaction was monitored by direct infusion into the mass spectrometer for 24 h at different times (0.5, 1, 2, 3, 4, 5, 10, and 24 h). At each time point, 100 μL reaction mixture was taken and added to 900 μL of 1:1 ratio of methanol/water, centrifuged for 2 min, and used for mass spectrometric (MS) analysis.

All experiments were done on a Waters Quatro Micro triple quadrupole mass spectrometer by using electrospray ionization in negative ion mode, with an electrospray ionization-MS capillary voltage of 3.0 kV, a cone voltage of 40 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 400 L/h. Cone gas flow was set at 60 L/h. Daughter ion spectrum was obtained using argon as collision gas. Desolvation temperature and source temperature were set to 200°C and 100°C, respectively. Electrospray ionization interference tuning and mass calibration were accomplished in the positive ion mode by using a standard sodium iodide-rubidium iodide solution. The sample was introduced to the source at a flow rate of 10 μL/min by using an inbuilt pump. The masses of parent ion and daughter ions were obtained in the MS and tandem MS operation.

**Statistical Considerations**

The association between genotypes and progression-free survival and overall survival was determined by the Kaplan-Meier method using a two-tailed log-rank test. The homzygous wild-type (CYP1B1*1/*1) and heterozygous variant (CYP1B1*1/*3) genotypes were pooled in the analysis based on similarity of predicted outcomes. The associated P was adjusted to be two times the unadjusted value to account for the two implicit comparisons made in determining the desirability of combining groups. Progression was censored at the off-study date if a patient was removed for toxicity or another reason.

**Results**

**Genotyping Results**

CYP1B1*3 variants were in Hardy-Weinberg equilibrium ($P = 0.57$). CYP1B1*3 genotyping data were compared with...
previously reported genotype frequencies in a predominantly Caucasian population (27) and found to be similar ($P = 0.42$, $\chi^2$ test). A summary of genotype information for each trial is provided in Table 2.

Pharmacokinetic Analysis

To address the question of whether CYP1B1 genetic variation is associated with docetaxel plasma concentrations, pharmacokinetic data from 23 patients treated with...
docetaxel (22) was analyzed as a function of CYP1B1*3 genotype. There was no statistically significant effect of genotype on the clearance of docetaxel (38.2 L/h, 95% confidence interval, 27.5-48.9 L/h in patients who are homozygous or heterozygous for the wild-type allele and 32.0 L/h, 95% confidence interval, 26.9-37.0 L/h in patients who are homozygous for the variant allele; \( P = 0.39 \), Mann-Whitney test). This is consistent with in vitro studies indicating that docetaxel is not metabolized by CYP1B1 (6).

**Genotype-Phenotype Relationships**

CYP1B1*3 genotype status was found to be a potential marker for a poor prognosis in patients who have undergone docetaxel-based treatment for AIPC (\( P = 0.0004 \), stratified by treatment received). The individuals with the wild-type sequence were combined with those carrying the heterozygote genotype and were found to have a significantly longer overall median survival (27.7 months) compared with that of patients who were homozygous variant for CYP1B1*3 (12.8 months; Fig. 1A). It should also be noted that the patients who received docetaxel alone or with prednisone exhibited nearly identical survival overall, whereas the survival probabilities were higher for patients who received docetaxel, estramustine, and thalidomide. In a combined group of patients who received docetaxel alone or with prednisone (Fig. 1B), the survival varied significantly by genotype (\( P = 0.016 \)) and the same effect was found in the group receiving the three drug combination (\( P = 0.018 \)), further strengthening the likelihood a true relationship exists between overall survival and genotypic expression of CYP1B1*3. This association was not observed between CYP1B1*3 and progression-free survival (\( P = 0.21 \), stratified by treatment).

To address whether the observed overall survival difference was attributable to docetaxel treatment but not when other therapies were used, the CYP1B1*3 allele was also assessed in men with AIPC treated with suramin-based therapy on three trials and thalidomide alone on a fourth (Fig. 2). There was no statistically significant association of the CYP1B1*3 genotype with overall survival in these patients (\( P = 0.58 \), stratified by the four treatment groups). Furthermore, in three of the four studies, the survivals were essentially identical in their lack of effect according to genotypic expression of CYP1B1*3, whereas in one study (suramin plus aminoglutethimide), there was only a trend toward an indication that a favorable outcome was more likely in the homozygous variant group (\( P = 0.086 \) after consideration of the genotype grouping used). These results further show that the influence of the CYP1B1*3 allele is associated with docetaxel treatment efficacy and is not related to an increased risk of disease progression in patients with AIPC as a whole.

**In vitro Tubulin Polymerization Assay**

A significant delay in the onset of taxane-induced tubulin polymerization was observed in the presence of 2-methoxyestradiol, a known microtubule inhibitor (28), whereas 4-methoxyestradiol was not different from the control. All

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Microtubule polymerization kinetics *in vitro*. A, docetaxel alone or docetaxel in combination with either podophyllotoxin, 2-methoxyestrogen, or 4-methoxyestrogen. B, docetaxel in combination with 2,3-quinone, or 3,4-quinone estrogen. C, paclitaxel alone or paclitaxel in combination with either podophyllotoxin, 2-methoxyestrogen, or 4-methoxyestrogen. D, paclitaxel in combination with 2,3-quinone or 3,4-quinone estrogen.
three groups were similar 500 s after initiation of polymerization. As predicted, tubulin polymerization was inhibited completely by the omission of docetaxel (data not shown) and also by podophyllotoxin, a known potent microtubule inhibitor (Fig. 3A; ref. 29). The 2,3-quinone caused a delay in onset of polymerization as well as a reduction in the final yield, whereas the 3,4-quinone totally inhibited polymerization, equivalent to podophyllotoxin (Fig. 3B). The structurally related taxane paclitaxel was less efficient at tubulin polymerization compared with docetaxel (tubulin polymerization kinetics achieved steady state at ∼250 s versus ∼100 s), although similar tubulin polymerization kinetics were observed with the addition of all of the aforementioned quinone compounds (Fig. 3C and D).

**Formation of Estrogen-3,4-Quinone Adduct with Docetaxel**

We have used MS to monitor the reaction of E2-3,4-quinone with docetaxel. The reaction was carried out at 37°C in phosphate buffer (pH 7). Under these conditions, the formation of the estrogen-docetaxel adduct was increased linearly up to 60 min and thereafter began to level off, indicating the reaction was completed within 1 h. The mass spectrum showed a molecular ion at m/z 1,094.2 [M + H]+ (Fig. 4A), clearly showing the formation of 4-OHE2-docetaxel adduct. The formation of the adduct was expected to be between the electrophilic C-1 position of E2-3,4-quinone and the nucleophilic nitrogen in the side chain of docetaxel (26). The structure determination of this adduct was confirmed by tandem MS. When the molecular ion at m/z 1,094.2 was fragmented, it showed the peaks at m/z 1,050, 806, 690, 550, 544, and 287 (Fig. 4A and B). The peak at 806 (docetaxel) is due to the loss of 287 (4-OHE2) from the molecular ion. The fragment ion at 544 and 550 represents the baccatin ring and the side chain linked to the 4-OHE2 moiety, respectively. Peak at 690 confirms the site of 4-OHE2 attachment at the nitrogen atom of the docetaxel side chain.

**Discussion**

This study provides evidence that CYP1B1 function may be linked to the clinical response to docetaxel treatment in
AIPC and suggests that this link is based on variations in catalysis and expression brought on by a common functional polymorphism in the CYP1B1 gene, that is, CYP1B1*3. This finding is independent of a potential influence of the CYP1B1*3 polymorphism on the systemic clearance of docetaxel and is therefore likely due to a gene-drug interaction wherein CYP1B1 catalysis influences docetaxel treatment efficacy.

This study shows that CYP1B1-catalyzed estrogen-3,4-quinone and methoxyestradiols could be responsible for the decreased efficacy of docetaxel through two possible mechanisms that are not mutually exclusive: (a) through an interference with the drug primary mechanism of action, microtubule stabilization, and (b) by structurally altering docetaxel. It was found that the quinones of the two catechol estrogen metabolites of CYP1B1, 2-OHE2 and 4-OHE2, both inhibited docetaxel- and paclitaxel-mediated tubulin polymerization. Whereas the quinone and methoxy derivatives of a minor CYP1B1 metabolite (2-OHE2) inhibited the nucleation and yield of polymerized tubulin, the quinone of the major CYP1B1 metabolite (4-OHE2) completely abrogated the polymerization of tubulin similar to podophyllotoxin. Interestingly, the CYP1B1*3 allele has been shown to be associated with an increased 4-OHE2/2-OHE2 ratio, and the E2-3,4-quinone is clearly more active in interfering with microtubule stabilization in the presence of the taxanes. Further, docetaxel reacts with E2-3,4-quinone at biological pH, indicating that the CYP1B1-catalyzed E2-3,4-quinone can also interfere with docetaxel efficacy by covalently binding the drug itself and presumably reducing potency.

Taxanes cause alterations in microtubule dynamics by promoting the formation of, and stabilizing, tubulin polymers. Therefore, it is likely that the effect of the E2-3,4-quinone in situ is to bind to tubulin monomers and prevent them from being incorporated into the microtubule, thereby minimizing the available pool of tubulin monomers below the critical concentration of protein required to initiate microtubule polymerization. Such binding could also alter the equilibrium of taxane-induced tubulin stabilization, such that depolymerization reactions would be more favored. Similar results have been shown when vinblastine (a microtubule destabilizing agent) was coadministered with paclitaxel (30). In this case, paclitaxel-induced tubulin polymerization was antagonized by vinblastine when the two drugs were administered together, whereas when vinblastine was administered 4 h following paclitaxel, tubulin polymerization was reversed. As estrogen quinones could be present at relatively high endogenous levels in prostate tissue (31), it is likely that these compounds act on tubulin polymerization by the former mechanism, which is by antagonizing microtubule stabilization by the taxanes.

The current data provide evidence that the CYP1B1*3 polymorphism is associated with a significant decrease in the overall survival following docetaxel treatment in patients with AIPC. Although the clinical pharmacogenetics study was conducted in a relatively small set of patients receiving docetaxel ($n = 52$), the data across the three different trials are consistent and suggest a large, highly significant decrease in docetaxel efficacy in patients carrying the CYP1B1*3/*3 genotype that is independent of interpatient variability in docetaxel drug metabolism. Furthermore, this significant decrease in survival is not observed in patients treated with non-taxane-based regimens, suggesting that the gene-drug interaction between CYP1B1 metabolism and docetaxel is driving the association between CYP1B1 genotype and survival. Recently, an association has also been shown between this polymorphism and a decreased progression-free survival following high-dose paclitaxel-based combination chemotherapy in patients with breast cancer (8). It is likely that the aforementioned mechanism of docetaxel resistance is responsible for the clinical observations in both studies, such that those patients carrying the CYP1B1*3 allele do not respond favorably to taxane therapy. However, cancer progression may also be influenced in the long term for those patients carrying the CYP1B1*3 allele due to the formation of genotoxic metabolites, such as E2-3,4-quinones. It is notable that in all cases those patients possessing a homozygous variant for the CYP1B1*3 polymorphism may exhibit a trend toward decreased overall survival in some patients following therapy. For example, even in our population of patients with AIPC treated with suramin alone or with other agents, or with thalidomide alone, with no overall statistical difference in survival, 14 of 112 individuals carrying the homozygous variant genotype survived longer than 5 years, whereas there were no 5-year survivors in patients with other genotypes. This is most likely attributable to the increased risk of progression due to the genotoxicity of the quinone estrogens, as they also react with purine nucleotides in the DNA causing genotoxicity (9).

Herein, we presented evidence that E2-3,4-quinone formed by CYP1B1 reduce the efficacy of docetaxel by both inhibiting tubulin polymerization and reacting with docetaxel. This study also serves as proof of concept that not only does CYP1B1-mediated estrogen metabolism increase the risk of developing prostate cancer (5) but it also interferes with the treatment of advanced prostate cancer, and potentially also other types of cancer, with taxanes. Whereas the current literature on CYP1B1 alleles in relation to treatment outcome with taxanes currently consists of relatively small numbers of patients, it is hoped that larger cohorts will verify these results and that eventually taxane-based therapies will be reconsidered in patients with hormone-mediated malignancies that also carry the CYP1B1*3 polymorphism.

References
CYP1B1 Metabolism Interferes with Taxane Treatment


Molecular Cancer Therapeutics

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