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ORIGINAL ARTICLE

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Increased activity of CYP3A enzyme in primary cultures of rat hepatocytes treated with docetaxel: comparative evaluation with paclitaxel

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Abstract *Purpose*: Docetaxel, a potent antimicrotubule agent widely used in the treatment of ovarian, breast and lung cancer, is extensively metabolized in various animal species, including humans. The metabolism of docetaxel to its primary metabolite, hydroxydocetaxel, is mediated by cytochrome P450 isozymes CYP3A2 and CYP3A4 in rats and humans, respectively. Several substrates of enzymes belonging to the CYP3A subfamily are known to induce different CYP isozymes, including CYP3A enzymes. Recently, paclitaxel, a compound structurally related to docetaxel, has been shown to significantly elevate the expression of CYP3A in rat and human hepatocytes. In this study we investigated the influence of docetaxel, employed at clinically relevant concentrations, on the level and the activity of cytochrome P450 3A in primary cultures of rat hepatocytes. *Methods*: Rat hepatocytes were treated with different concentrations of docetaxel, paclitaxel and other CYP3A inducers. Testosterone 6β-hydroxylase activity of intact hepatocytes was used as a marker for CYP3A. The immunoreactive CYP3A levels in the S-9 fractions were determined by Western blot analysis. *Results*: We observed that by day 3 of drug treatment, docetaxel at concentration in the range of 2.5–10 μM increased the CYP3A enzymatic activity and the immunoreactive CYP3A levels in a concentration-dependent manner. At the 10 μM level, docetaxel caused a twofold increase in the CYP3A activity and a threefold increase in the immunoreactive CYP3A levels. However, the docetaxel-mediated CYP3A activity and enzyme level increase were significantly lower than those mediated by paclitaxel and dexamethasone. A comparison of the testosterone 6β-hydroxylation activity in hepatocytes treated with these agents at a concentration of 5 μM each yielded the following rank order of induction capacity: dexamethasone > paclitaxel > docetaxel (15-fold, 5-fold, 2.2-fold, respectively). *Conclusions*: Taken together, our findings raise the possibility that docetaxel at clinically relevant concentrations increases CYP3A activity. The potential for docetaxel-mediated changes in the metabolism of other coadministered drugs and its own metabolism, in relation to that due to paclitaxel, are discussed.

Keywords Docetaxel · Cytochrome P450 · CYP3A · Hepatocyte · Activity

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Introduction

Paclitaxel (Taxol) and docetaxel (Taxotere), two members of the novel class of antitumor agents, taxanes, have emerged in the last two decades as effective drugs in the treatment of a variety of malignancies. Paclitaxel is a semisynthetic taxane isolated from the bark of the Pacific yew tree *Taxus brevifolia* [26]. Docetaxel is a semisynthetic taxane isolated from the needles of the European yew *Taxus baccata* [6]. The taxanes have a unique mechanism of action acting as promoters of microtubule polymerization [9, 22]. This leads to a variety of effects on dividing cells, including cell cycle arrest in the G_2/M phase, apoptosis and acute cytotoxicity. Both paclitaxel and docetaxel are widely used in the treatment of ovarian, breast, non-small-cell lung and head and neck cancers. The effectiveness of these agents in the treatment of other malignancies and the development of other more promising taxanes continue to be investigated.

Metabolism and pharmacokinetic investigations have established that both paclitaxel and docetaxel are

metabolized predominantly via cytochrome P450-dependent hepatic metabolism. The principal site of docetaxel metabolism is the *tert*-butyl group at the C_{13} side chain to form hydroxydocetaxel (M2) (Fig. 1). This metabolite undergoes further oxidation and cyclization [7, 17, 21]. There is little interspecies variability in docetaxel metabolism and the conversion of docetaxel to hydroxydocetaxel, mediated by CYP3A subfamily, occurs in both rats and humans [17, 21]. Paclitaxel metabolism in humans primarily involves conversion of paclitaxel to 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel by CYP2C8 and CYP3A4, respectively. CYP3A4 is further involved in the conversion of 6α-hydroxypaclitaxel to 6α-3′-*p*-dihydroxypaclitaxel [5, 8, 13, 18, 20]. 6α-Hydroxypaclitaxel is considered to be the most abundant metabolite in humans. However, there is considerable interindividual variability in paclitaxel metabolism, and in some subjects, especially those pretreated with known enzyme inducers such as barbiturates, CYP3A-driven metabolites are present in high levels [23]. In contrast to docetaxel, there is marked interspecies variation in paclitaxel metabolism. For example, 6α-hydroxypaclitaxel is not detected in rat tissue [24].

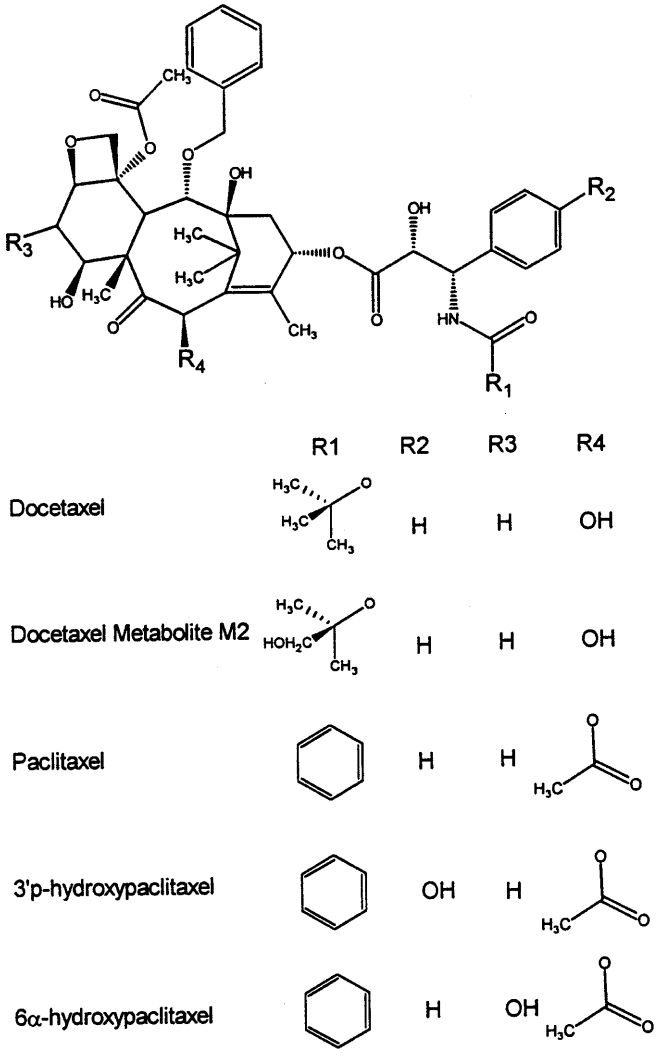

Fig. 1 Structure of paclitaxel, docetaxel and their respective metabolites

A feature shared by several CYP3A substrates is that these compounds also have the ability to induce CYP3A and other enzymes. Accordingly, recent studies have shown that paclitaxel is a potent inducer of CYP3A enzymes in primary cultures of rat and human hepatocytes [11, 12]. Since anticancer compounds such as taxanes are used in combination with a number of other therapeutic agents and adjuvants, many of which are CYP3A substrates, enzyme induction could potentially result in clinically significant alteration in the metabolism of other compounds as well as their own metabolism. The possibility that other taxanes, especially docetaxel, have the potential to act as enzyme inducers has not been investigated. In this study, we employed primary cultures of rat hepatocytes for a comparative evaluation of docetaxel and paclitaxel as CYP3A inducers. Our studies showed that at clinically relevant concentrations [3, 4, 15], docetaxel significantly increased the activity and immunoreactive levels of CYP3A in primary rat hepatocyte cultures. However, on an equimolar basis, the magnitude of the paclitaxel-mediated induction appeared to be much higher than that due to docetaxel.

Materials and methods

Chemicals and reagents

Docetaxel was a gift from Aventis Pharmaceuticals (Collegeville, Pa.). Paclitaxel, dexamethasone, phenobarbital, testosterone and 6β-hydroxytestosterone were obtained from Sigma Chemical Co. (St. Louis, Mo.). William's E culture medium (HMM) and medium supplements, dexamethasone, gentamicin and insulin, were obtained from BioWhittaker (San Diego, Calif.). Polyclonal antibody for rat CYP3A raised in goat was obtained from Gentest (Woburn, Mass.) and horseradish peroxidase-conjugated anti-goat secondary antibodies were obtained from Oxford Biomedical Research (Oxford, Mich.). The ECL chemiluminescence detection system was obtained from Amersham Pharmacia Biotech (Piscataway, N.J.).

Hepatocyte culture

Rat hepatocytes were obtained from Cedra Corporation (Austin, Tx.). Hepatocytes (1×10^6 cells/well) were obtained in collagen-coated six-well plates. Liver tissues from healthy male Sprague-Dawley rats weighing approximately 260 g were used for isolating the hepatocytes. Viability at plating, measured using trypan blue exclusion, was at least 94%. Upon receipt (24 h after plating), the rat hepatocytes were maintained in William's E medium supplemented with insulin (10^{-7} *M*), dexamethasone (10^{-7} *M*) and gentamicin (0.5 μg/ml) at 37°C in an atmosphere containing 5% CO_2 in an incubator. The cells were allowed to acclimatize for 1–2 h prior to initiating the experiments.

Drug treatment

Following the acclimatization period, the hepatocyte culture medium was removed and replaced with fresh medium supplemented

with test compounds. Hepatocytes were treated with docetaxel (0.5–50 μ*M*), paclitaxel (5–10 μ*M*) and dexamethasone (5 μ*M*) for 24–72 h. Stock solutions (1000×) of paclitaxel, docetaxel and dexamethasone were prepared in dimethyl sulfoxide. Control hepatocytes received equivalent amounts of DMSO (final concentration 0.1%). The drug-containing medium was changed every 24 h. Following drug treatment, cells were processed for measurement of immunoreactive protein content or enzymatic activity.

Assay for CYP3A activity

Testosterone 6*β*-hydroxylase activity of intact cultured hepatocytes was employed as a marker for CYP3A activity [1]. After a treatment period of 24–72 h, the culture medium was replaced with fresh William's E medium for 10–15 min of incubation. The medium was then replaced with William's E medium (1 ml/plate) containing testosterone (250 μ*M*) followed by incubation for 15 min. To the culture medium (500 μl) was added 11α-hydroxyprogesterone (2.5 μg) as an internal standard. The medium was then extracted with 5 ml dichloromethane and the solvent was evaporated under nitrogen. The samples were reconstituted in 1:1 v/v methanol/water and injected into a 10-μm μ-Bondapak C_{18} column (3.9×300 mm) with a 10-μm μ-Bondapak C_{18} guard column. The 6*β*-hydroxytestosterone levels were measured using a modification of a previously reported reversed-phase HPLC method [1]. Briefly, this entailed using a concave gradient procedure starting from 90% solvent A (methanol/water/acetonitrile 39:60:1) to 85% solvent B (methanol/water/acetonitrile 80:18:2) over 22 min. The gradient with 85% solvent B was maintained for an additional 8 min. This was followed by reverting to the initial solvent condition (90% solvent A) over 5 min, and this was maintained for another 5 min. A Waters 486 UV/VIS detector at a wavelength of 240 nm was used for the detection of 6*β*-hydroxytestosterone. The total run lasted for 40 min, with 6*β*-hydroxytestosterone, 11α-hydroxyprogesterone and testosterone eluting at 19.3, 25.9, and 28.1 min, respectively.

Immunodetection of CYP3A protein

Control and treated hepatocytes were processed for the preparation of S-9 fractions. Cells were scraped from the wells and resuspended in 0.1 *M* potassium phosphate buffer (pH 7.6), and homogenized using a tissue homogenizer. Homogenates were centrifuged at 9000 *g*, and the resulting supernatant (S-9 fraction) was stored at –80°C until use. Protein concentrations were determined using a modification of the Bradford protein assay (Bio-Rad assay) [2]. The S-9 fractions (1 μg protein) were resolved by SDS-PAGE (12% acrylamide) and electrophoretically transferred to nitrocellulose membrane [14]. The nitrocellulose membranes were blocked with 3% bovine serum albumin in PBST (0.1 *M* sodium phosphate buffer, pH 7.4, containing 0.1% Tween 20) for 45 min. The membranes were probed for immunoreactive CYP3A with a polyclonal anti-rat CYP3A antibody (1:500) for 1 h. The antibody binding was visualized using a horseradish peroxidase-conjugated anti-goat secondary antibody (1:5000), followed by enhanced chemiluminescence detection (ECL, Amersham). The immunoblots were quantitated with a NucleoVision image analyzer using Gel Expert photodensitometry software (Nucleotech, Calif.). The polyclonal anti-rat CYP3A antibody recognizes both CYP3A1 and CYP3A2 isozymes in rat.

Statistical analysis

The formation of 6*β*-hydroxylation of testosterone and the fold increases in immunoreactive CYP3A levels in various treatment groups were analyzed by a two-factor ANOVA, followed by a *t*-test to compare the mean values in control and drug-treated rat hepatocytes. $P < 0.05$ was interpreted as the level of statistical significance.

Results

To investigate the concentration and time dependence of increases in CYP3A activity and immunoreactive levels, primary cultures of rat hepatocytes were treated for 24–72 h with graded docetaxel levels (0.1–50 μ*M*). Paclitaxel and dexamethasone, known CYP3A inducers, were used as controls. The CYP3A activity was measured by determining the ability of intact hepatocytes to convert testosterone to 6*β*-hydroxytestosterone. For immunodetection of CYP3A protein, cells were harvested and processed for preparation of S-9 fractions. The immunoreactive levels were detected using Western blot analysis. A typical HPLC profile of testosterone, 6*β*-hydroxytestosterone and 11α-hydroxyprogesterone (internal standard) obtained from the cell culture medium of untreated control hepatocytes and hepatocytes treated with docetaxel (5 μ*M*) is shown in Fig. 2.

Increase in CYP3A activity in hepatocytes was a function of both drug concentration and treatment period. In general, neither docetaxel nor paclitaxel increased the CYP3A activity within 24 h (data not shown). By 48 h paclitaxel (10 μ*M*) had caused a twofold increase, while docetaxel (0.1–50 μ*M*) did not significantly ($P > 0.19$) alter the CYP3A activity (Fig. 3). However, by 72 h docetaxel had caused a concentration-dependent increase in the CYP3A activity. At concentrations of 2.5–10 μ*M*, there was a statistically significant ($P < 0.01$) increase in the activity, resulting in 1.7- to 2-fold increase in 6*β*-hydroxylation of testosterone. The increase in enzymatic activity in response to paclitaxel (10 μ*M*) treatment was markedly higher resulting in a 4-fold increase ($P = 0.01$; Fig. 4).

The immunoblot and fold increases in the immunoreactive CYP3A levels in drug-treated compared to control hepatocytes for the treatment period 72 h are shown in Fig. 5A and B, respectively. The pattern of response as a function of docetaxel concentration was very similar to that observed for enzyme activity. Again, differences in the expression of CYP3A in treated vs control hepatocytes were observed only at docetaxel concentrations above 2.5 μ*M*. At docetaxel concentrations in the range 2.5–10 μ*M*, CYP3A levels were two- to threefold ($P < 0.04$) higher than those in control hepatocytes. There was a very good correlation ($r^2 = 0.9152$) between the increase in the immunoreactive CYP3A levels and CYP3A activity measured as 6*β*-hydroxylation of testosterone in rat hepatocytes treated with various concentrations of docetaxel (0.5–10 μ*M*; Fig. 6). Paclitaxel (10 μM) caused a sevenfold ($P = 0.02$) increase in the immunoreactive CYP3A levels.

Since dexamethasone is a known potent CYP3A inducer in rat hepatocytes, in a separate set of experiments we also compared the increase in CYP3A activity and enzyme levels in response to docetaxel and paclitaxel treatment with that in response to dexamethasone. The results of Western blot analysis and testosterone 6*β*-hydroxylation activity following a 72-h treatment period

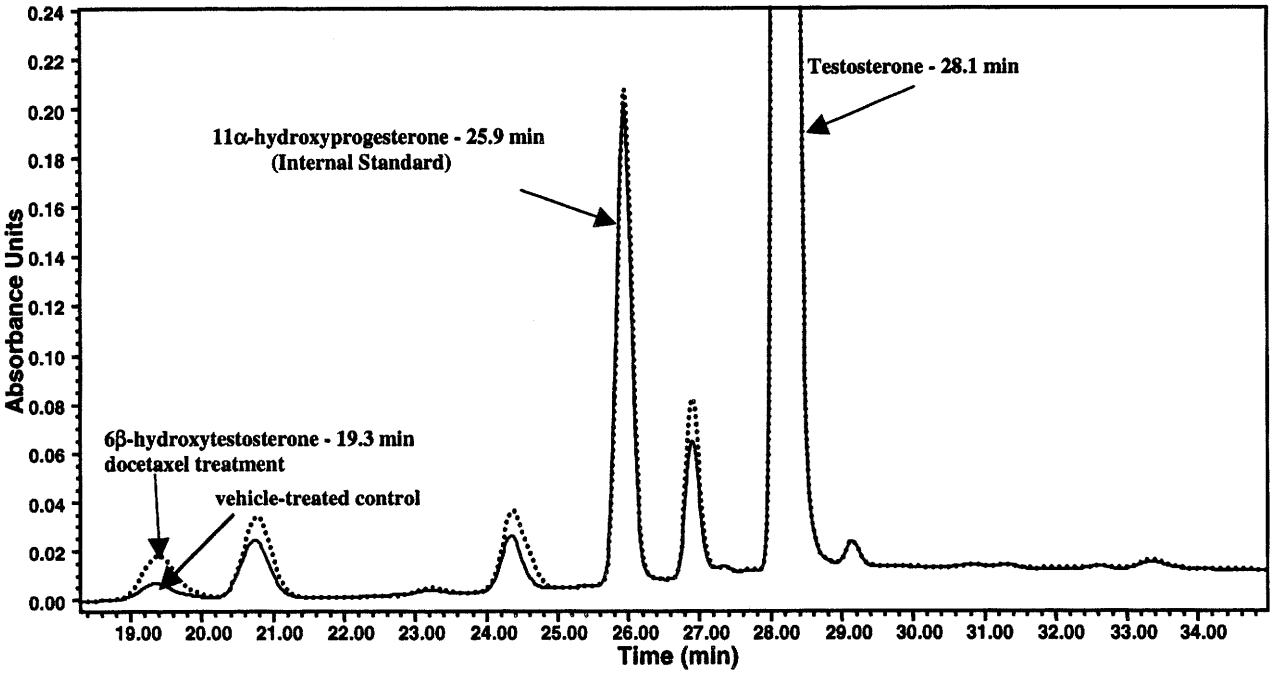

Fig. 2 A typical HPLC profile obtained from a dichloromethane extract of the culture medium of hepatocytes incubated with testosterone (250 μ*M*) for 15 min (*solid line*, untreated control hepatocytes, *dotted line*, hepatocytes treated with 5 μ*M* docetaxel). The retention times for 6*β*-hydroxytestosterone, 11*α*-hydroxyprogesterone (internal standard) and testosterone were 19.3, 25.9 and 28.1 min, respectively

are shown in Fig. 7A, B, and Fig. 8, respectively. The increase in the immunoreactive CYP3A levels caused by paclitaxel (5 μ*M*) and dexamethasone (5 μ*M*) was 9-fold ($P = 0.0008$) and 14.5-fold ($P = 0.003$), respectively. Docetaxel, however, caused only a 2.6-fold ($P = 0.04$) increase in CYP3A levels (Fig. 7A, B). Comparison of CYP3A activities indicated that both paclitaxel and docetaxel were markedly less effective than dexamethasone. While docetaxel (5 μ*M*) and paclitaxel (5 μ*M*) caused a 2.2- ($P = 0.02$) and a 5-fold ($P < 0.001$) increase in enzyme activity, respectively, dexamethasone (5 μ*M*) caused a 15-fold ($P < 0.001$) increase (Fig. 8).

Discussion

Docetaxel at clinically relevant concentrations considerably elevated the immunoreactive levels of CYP3A enzymes in rat hepatocytes and this increase was associated with enhanced metabolism of testosterone to 6*β*-hydroxytestosterone. The increase in CYP3A activity and enzyme levels was a function of docetaxel concentration and treatment period. With a 72-h treatment period, a 2- to 3-fold increase in CYP3A levels and a 1.7- to 2-fold increase in 6*β*-hydroxylation activity at docetaxel concentrations of 2.5–10 μ*M* were observed. This is the first report of a docetaxel-mediated increase of hepatic CYP enzymes. As indicated earlier, previous

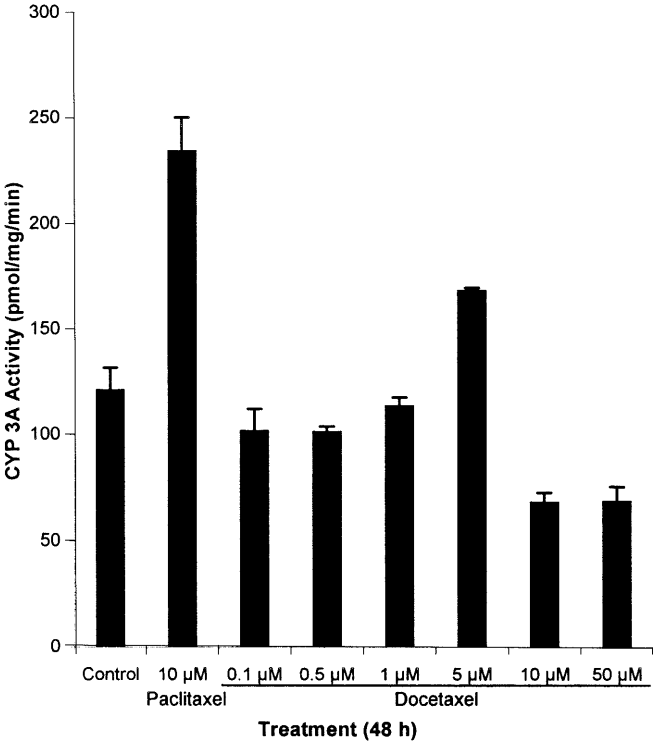

Fig. 3 CYP3A activity measured as 6*β*-hydroxylation of testosterone in rat hepatocytes treated for 48 h with docetaxel (0.1–50 μ*M*) and paclitaxel (10 μ*M*) compared to that in untreated control hepatocytes (means ± SEM, $n = 2$). Following drug treatment the rat hepatocytes were incubated with culture medium containing testosterone (250 μ*M*) for 15 min at 37°C. The culture medium was analyzed for 6*β*-hydroxytestosterone as described in Materials and methods using a reversed-phase HPLC method

studies have shown that paclitaxel induces CYP3A enzymes in primary cultures of rat and human hepatocytes. The fold increase in the immunoreactive levels of

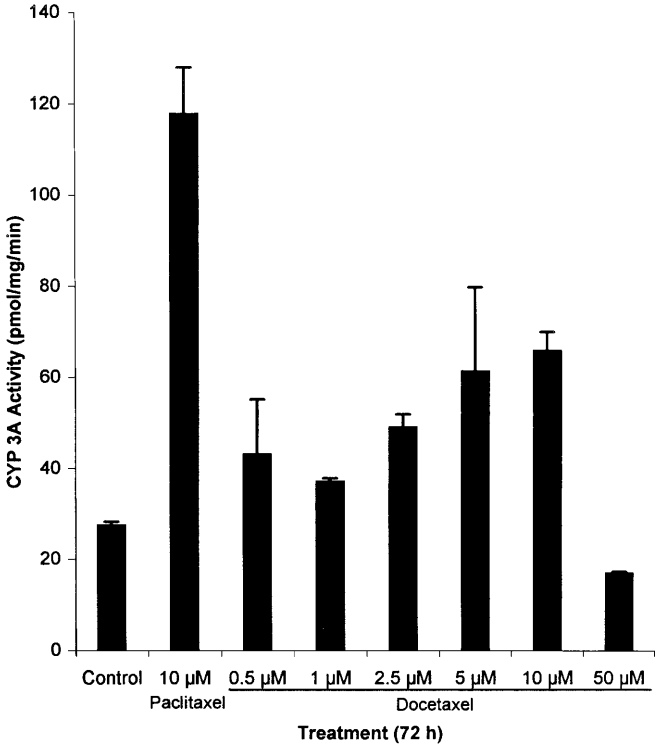

Fig. 4 CYP3A activity measured as 6β-hydroxylation of testosterone in rat hepatocytes treated for 72 h with docetaxel (0.5–50 μM) and paclitaxel (10 μM) compared to that in untreated control hepatocytes (means ± SEM, $n = 2$). Following drug treatment the rat hepatocytes were incubated with culture medium containing testosterone (250 μM) for 15 min at 37°C. The culture medium was analyzed for 6β-hydroxytestosterone as described in Materials and methods using a reversed-phase HPLC method

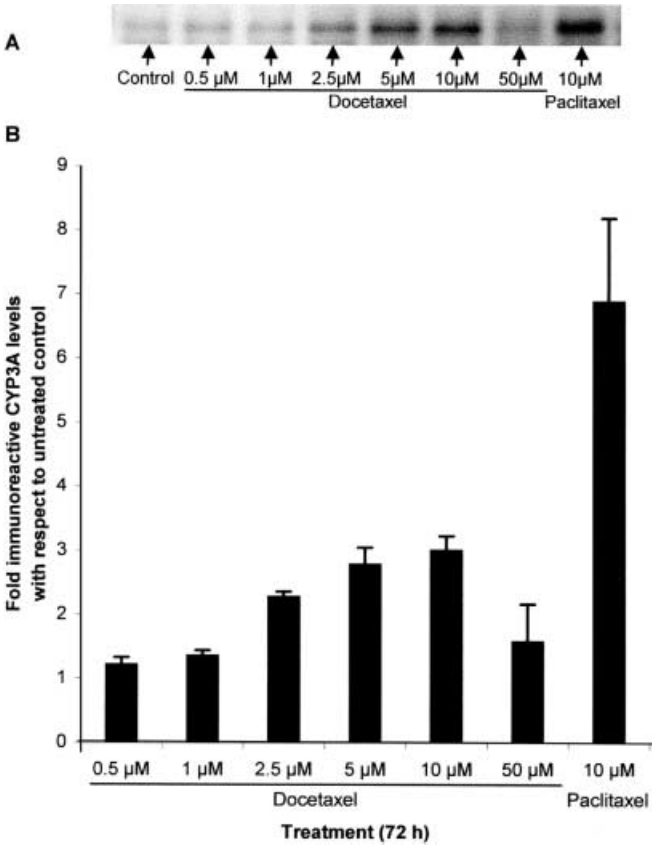

Fig. 5A, B Western blot analysis of rat hepatocytes showing the effects of treatment with docetaxel (0.5–50 μM) and paclitaxel (10 μM) on the levels of immunoreactive CYP3A. **A** Representative immunoblot showing immunoreactive CYP3A expression following various treatments. The S-9 fractions of the drug-treated and untreated control hepatocytes were resolved electrophoretically as described in Materials and methods. The immunoreactive CYP3A protein was probed with an anti-rat CYP3A primary antibody and visualized using a horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence reaction. Two separate immunoblots were developed from duplicates of the various treatments. **B** Photodensitometric analysis of the two immunoblots showing the fold increases in the immunoreactive CYP3A levels in the drug-treated rat hepatocytes with respect to the untreated control hepatocytes (means ± SEM, $n = 2$)

CYP3A in rat hepatocytes treated with paclitaxel (10 μM) observed in this study (7-fold) was somewhat lower than the 8.5-fold reported by Kostrubsky et al. [11]. This may have been due to differences in the experimental design. In their experiments, Kostrubsky et al. [11] allowed a period of 48 h after plating the hepatocytes before challenging them with paclitaxel and other inducers for a 48-h treatment period, while in our studies cells were treated 24 h after plating for a period of 72 h.

Since rat hepatocytes were used in this study, further studies, especially using human hepatocytes, will certainly be required to elucidate the clinical relevance of our findings. These findings do, however, indicate the possibility that induction of CYP enzymes by docetaxel could have clinical implications. The peak plasma levels of docetaxel following the usual doses of 75–100 mg/m^2 infused over 1 h are in the range 1–8 μM, with median concentrations in the range 2–3 μM [4, 15]. Therefore, our observation of an increase in CYP3A levels at docetaxel concentrations as low as 2.5 μM underscores the clinical relevance of our findings. Further, extrapolation of our results to the in vivo situation is complicated by several factors. For assessing the potential for docetaxel-mediated induction of CYP3A in vivo, several other factors, such as hepatic drug uptake and differences in the docetaxel elimination half-life in our experimental system vs those in vivo, need to be considered.

Highly lipophilic compounds such as the taxoids have extensive tissue distribution, with the highest concentrations found in the hepatobiliary system. It has been previously reported that paclitaxel levels in the rat liver could be as much as 160-fold higher than in plasma [16]. Thus, it is likely that hepatocyte levels of docetaxel could also significantly exceed those used in our study, underscoring the potential for enzyme induction in vivo. However, an argument against this potential is that the drug levels represented in the cell culture medium in our model system are unlikely to be sustained in vivo for the 2–3 days that would be required for the induction of CYP3A activity. In our model system, the drug-containing cell culture medium was replaced every 24 h and

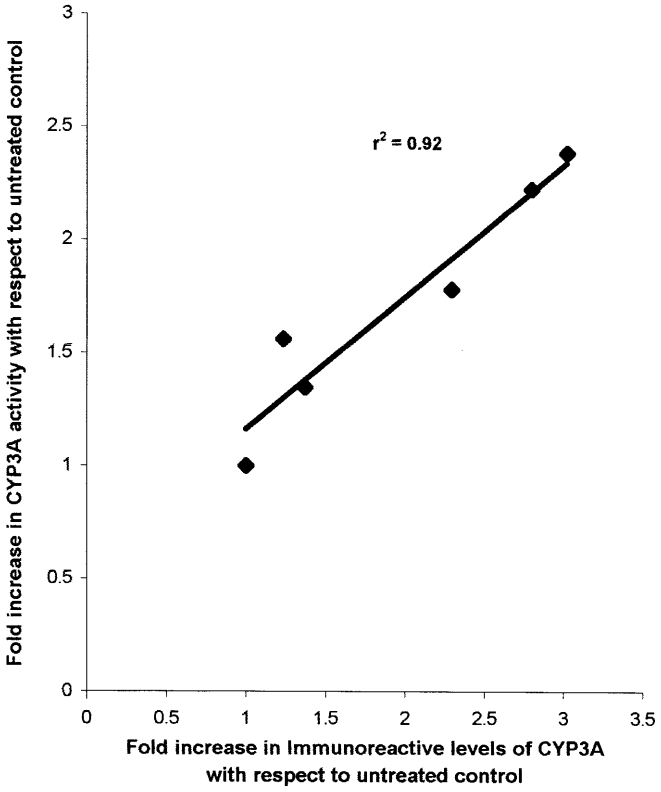

Fig. 6 Correlation between fold increase in immunoreactive CYP3A levels and CYP3A activity in rat hepatocytes treated with docetaxel (0.5–10 μ*M*). The fold increases in immunoreactive CYP3A levels (means, $n=2$) are plotted in relation to the fold increases in CYP3A activity measured as 6β-hydroxylation of testosterone (means, $n=2$)

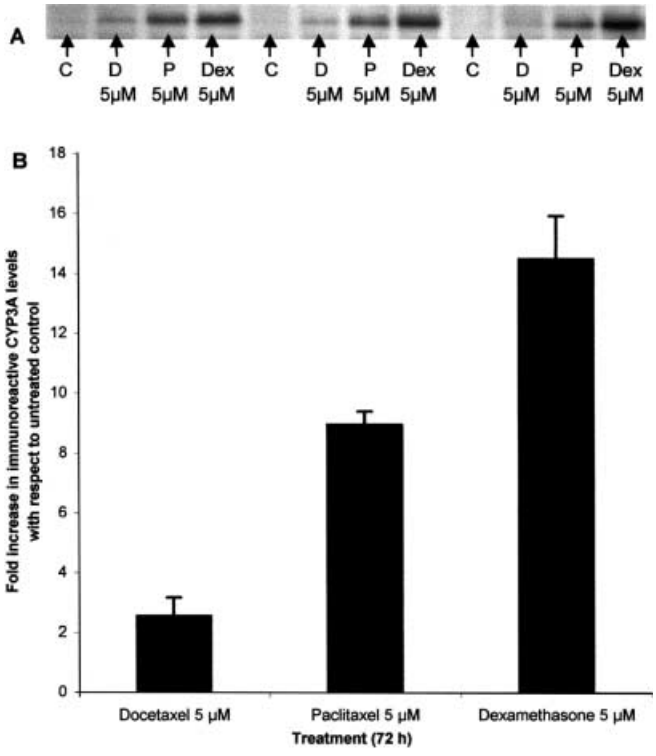

Fig. 7 **A** Western blot analysis of rat hepatocytes treated with docetaxel (*D*) (5 μ*M*), paclitaxel (*P*) (5 μ*M*) and dexamethasone (*Dex*) (5 μ*M*) on the levels of immunoreactive CYP3A compared to untreated control hepatocytes (*C*). The S-9 fractions of the drug-treated and untreated control hepatocytes were resolved electrophoretically as described in Materials and methods. The immunoreactive CYP3A protein was probed with an anti-rat CYP3A primary antibody and visualized using a horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence reaction. **B** Photodensitometric analysis of the immunoblot showing the fold increases in the immunoreactive CYP3A levels in the drug-treated rat hepatocytes with respect to the untreated control hepatocytes (means ± SEM, $n=3$)

although we would expect the docetaxel levels in the medium to decrease due to its metabolism, this may not mimic the in vivo situation. While there is significant interindividual variability in the observed plasma elimination half-lives of taxanes, the median range for docetaxel elimination half-life is 4–6 h. It is therefore likely that the plasma docetaxel levels could decrease to less than 10% of the peak levels within 24 h. However, the pattern of drug elimination from plasma may not mirror the decay over time of hepatic docetaxel levels, which is currently not known. These factors taken together suggest that it is likely that the intrahepatocyte docetaxel may be sustained for the period of time required for induction of CYP3A.

Both paclitaxel and docetaxel are usually coadministered with a number of anticancer drugs and adjuvants that are known CYP3A substrates. Examples of such anticancer compounds include etoposide, cyclophosphamide, doxorubicin, vinca alkaloids and tamoxifen [10]. A variety of other drugs include steroids such as dexamethasone and antiemetic agents such as granisetron and ondansetron. Thus, it is possible that docetaxel and paclitaxel could cause increased metabolism of some of these compounds. However, since the magnitude of paclitaxel-mediated induction was much higher than that of docetaxel, and considering that the induction of CYP3A activity by paclitaxel appears to require a shorter treatment period, the likelihood of such interactions would appear to be higher for paclitaxel than for docetaxel. In this regard, the potential for increased clearance of CYP3A4 substrates by paclitaxel was demonstrated in a recent study [19] in which an interaction between paclitaxel and SU5416, an antiangiogenic compound metabolized by CYP3A4 and CYP1A2, was found. It has been observed that the clearance of SU5416 in subjects who have received paclitaxel is much higher than in subjects who have not [19].

It is not clear if the observed CYP3A induction response is mediated by docetaxel, its metabolites or by a combination of these species. In the case of paclitaxel, since its primary metabolite 6α-hydroxypaclitaxel is not formed in rat hepatocytes, it is likely that the induction was primarily due to paclitaxel. Further studies are needed to explore this issue. This is an important concern since the concentrations of docetaxel metabolites in plasma and hepatocytes as well as their pharmacokinetic parameters are likely to be different from those of the

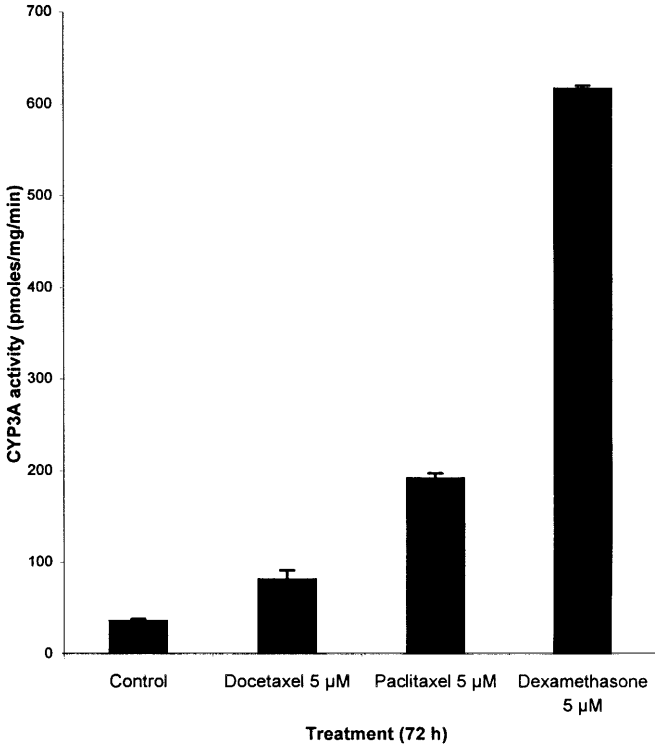

Fig. 8 CYP3A activity measured as 6β-hydroxylation of testosterone in rat hepatocytes treated for 72 h with docetaxel (5 μM), paclitaxel (5 μM) and dexamethasone (5 μM) compared with untreated control hepatocytes (mean ± SEM, $n = 3$). Following drug treatment the hepatocytes were incubated with culture medium containing testosterone (250 μM) for 15 min at 37°C. The culture medium was analyzed for 6β-hydroxytestosterone as described in Materials and methods using a reversed-phase HPLC method

parent compound. To the best of our knowledge, the pharmacokinetics of docetaxel metabolites in humans have not been reported, but it is known that paclitaxel metabolites have a longer elimination half-life than paclitaxel [25]. Thus, depending on the fraction of docetaxel dose converted to the metabolites and the elimination half-lives of the metabolites, the contribution made by the metabolites to the induction response could be significant.

In conclusion, this study demonstrated that docetaxel induced CYP3A levels and its activity in rat hepatocytes. The magnitude of the induction by docetaxel in this experimental model was lower than that of paclitaxel and dexamethasone. Further studies will clarify the clinical implications of docetaxel-mediated CYP3A induction on the metabolism of coadministered drugs or its own metabolism.

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