Effect of the St. John's Wort Constituent Hyperforin on Docetaxel Metabolism by Human Hepatocyte Cultures

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Abstract

Background and Purpose: St. John's wort is a commonly used herbal medication that increases cytochrome *P*450 3A (CYP3A) activity. Because docetaxel is inactivated by CYP3A, we studied the effects of the St. John's wort constituent hyperforin on docetaxel metabolism in a human hepatocyte model.

Experimental Design: Hepatocytes, isolated from three donor livers, were exposed to hyperforin (0.1, 0.5, or 1.5 μmol/L) or rifampin (10 μmol/L) for 48 hours. After 48 hours, hyperforin- or rifampin-containing medium was replaced with medium containing 100 μmol/L docetaxel. After 1 hour, docetaxel metabolism was characterized by liquid chromatography-tandem mass spectrometry. Subsequent incubations characterized the specific cytochrome *P*450s that produced the docetaxel metabolites observed in hepatocyte incubations.

Results: Rifampin induced docetaxel metabolism 6.8- to 32-fold above docetaxel metabolism in control cultures. Hyperforin induced docetaxel metabolism in all three hepatocyte preparations. Hyperforin induction was dose-dependent and, at maximum, was 2.6- to 7-fold greater than that in controls. Docetaxel metabolites identified in rifampin- and hyperforin-treated hepatocyte preparations included the previously described *tert*-butyl – hydroxylated metabolite and two previously unidentified metabolites involving hydroxylation on the baccatin ring. CYP3A4 produced the *tert*-butyl – hydroxylated metabolite and the two ring-hydroxylated metabolites. CYP2C8 produced one of the newly described ring-hydroxylated metabolites.

Conclusions: Exposure to the St. John's wort constituent hyperforin induces docetaxel metabolism *in vitro*. This implies that subtherapeutic docetaxel concentrations may result when docetaxel is administered to patients using St. John's wort on a chronic basis. The results also show induction of previously undescribed metabolic pathways for docetaxel, one of which may be analogous to the known 6-α-hydroxylation of paclitaxel by CYP2C8.

In 2002, sales of botanical supplements in the United States reached nearly $293 million. St.John' s wort accounted for $15 million in sales, making it the seventh highest grossing botanical supplement (1). Several clinical studies have claimed St. John's wort to be as effective as conventional therapy in the treatment of mild-to-moderate, but not severe, depression (2–6).

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Marketed St. John's wort, an extract of the flowering portion of the plant *Hypericum perforatum* L., is a mixture of a number of biologically active, complex compounds. At 0.3 mg per capsule, the naphthodianthrone hypericin is used as a means of standardizing the marketed product. The phloroglucinol hyperforin, the most plentiful lipophilic compound in the extract, is a potent inhibitor of serotonin, norepinephrine, and dopamine reuptake (7).

Several recent reports have documented decreased blood and plasma concentrations of cytochrome *P*450 3A4 (CYP3A4) substrates, such as indinavir, cyclosporine A, and imatinib, in patients concomitantly taking St. John's wort (8–11). Similar observations have been documented for digoxin, a substrate of the intestinal transporter P-glycoprotein. Additional *in vivo* evidence has shown that St. John's wort increased CYP3A4 and P-glycoprotein levels in rats (12). In primary human hepatocyte cultures, hyperforin, but not hypericin, was shown to induce CYP3A and CYP2C9 expression and activity with no effect on other common drug-metabolizing enzymes (13). Other investigators have suggested that chronic use of St. John's wort can also induce CYP1A2 and CYP2C19 *in vitro* (14) and CYP1A2 *in vivo* in females (15).

Both CYP3A4 and P-glycoprotein are transcriptionally regulated by the nuclear orphan receptor pregnane X receptor

(16–19). After ligand binding in the cytosol, pregnane X receptor translocates to the nucleus where it heterodimerizes with the retinoid X receptor and then binds to the *CYP3A4* promoter, resulting in increased expression of CYP3A4 and P-glycoprotein mRNA. *In vitro* studies have shown that hyperforin, but not hypericin, is a potent activator of pregnane X receptor (20, 21). In addition to increasing expression of CYP3A4 and P-glycoprotein, pregnane X receptor has also been shown to play a role in cytochrome P450 2C9 (CYP2C9) expression (22).

Docetaxel is a taxane antineoplastic agent with a broad spectrum of antitumor activity (23–25) and a mechanism of action that involves abnormal polymerization of tubulin with resultant mitotic arrest (26, 27). Unlike paclitaxel, which is metabolized primarily by CYP2C8 (28–32), docetaxel is metabolized primarily by CYP3A4 (32, 33). Moreover, clinical studies have shown a correlation between docetaxel clearance and the dose-limiting neutropenia resulting from its use (34, 35). Specifically, patients with impaired hepatic function and decreased CYP3A4 activity experience greater myelosuppression than do patients with normal hepatic function and CYP3A4 activity (34, 35). To date, studies examining the effect of induction of CYP3A4 activity on docetaxel metabolism and its clinical pharmacodynamic effects have not been reported. As an initial approach to this question, we used primary human hepatocyte cultures to characterize the effect of hyperforin on docetaxel metabolism.

Materials and Methods

Chemicals and reagents. Williams' E culture medium and the medium supplements, dexamethasone and insulin, were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin was obtained from Life Technologies (Grand Island, NY). Amphotericin B and bovine calf serum were obtained from Cambrex (Walkersville, MD). Rifampin and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). Hyperforin was isolated from St. John's wort leaf/flower mixtures at the National Center for Toxicological Research (Jefferson, AR). Purified hyperforin was identified by liquid chromatography-mass spectrometry (LC/MS) and nuclear magnetic resonance analysis, and the purity (>98%) was further determined by a LC photodiode array method (36). Falcon six-well culture plates were obtained from Becton Labware (Franklin Lakes, NJ). Human CYP3A4 + *P*450 reductase SUPERSOMES, CYP2C8 + *P*450 reductase + cytochrome b_5 SUPERSOMES, and CYP2C9*1 (Arg^{144}) + *P*450 reductase SUPERSOMES were purchased from GENTEST (BD Biosciences, Woburn, MA). All solvents and other chemicals used were of high-performance LC grade or the highest purity available.

Hepatocyte incubations. Hepatocytes were isolated from three human liver donors. Human liver tissue was procured under an Institutional Review Board–approved protocol and with support from the liver tissue procurement and distribution system. Donor information is summarized in Table 1. Hepatocytes were prepared by a three-step collagenase perfusion technique (37). Initial viability was >75% in all preparations.

Hepatocytes were plated on Falcon six-well culture plates (1.5×10^6 cells) or P100 (1×10^7 cells) plates, previously coated with rat tail collagen in 1.5 mL Williams' E medium supplemented with 0.1 μmol/L insulin, 0.1 μmol/L dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B, and 10% bovine calf serum. After 4 hours, during which cells were allowed to attach, medium was replaced with 1.5 mL serum-free medium containing all of the other supplements described above. Cells were maintained in culture at 37°C in an atmosphere containing 95% humidity, 5% CO_2, and 95% air. After 24 hours in culture, unattached cells were removed by gentle agitation and medium was replaced with 1.5 mL of fresh medium every 24 hours. Between 48 and 96 hours, cells were exposed to medium containing rifampin (10 μmol/L) or hyperforin (0.1, 0.5 or 1.5 μmol/L), each of which was dissolved in DMSO. Control cultures were exposed to medium containing DMSO alone. The final concentration of DMSO in all media was 0.1%. At 96 hours, cells were washed with 1.5 mL fresh medium for 1 hour and then incubated in 1.5 mL medium containing 100 μmol/L docetaxel for an additional hour. After the incubation with docetaxel, 0.75 mL of medium was removed and stored at −80°C until assayed for docetaxel metabolism.

Cytochrome P450 incubations. Individual cytochrome *P*450s (20 μL) were added to microcentrifuge tubes and incubated in a total volume of 200 μL containing 50 mmol/L sodium-potassium phosphate buffer (pH 7.4). 1.5 mmol/L NADPH, and 100 μmol/L docetaxel. After 30-minute incubation at 37°C in a shaking incubator, cytochrome *P*450 incubation mixtures were assayed by LC-tandem mass spectrometry (LC/MS/MS) as described below.

Liquid chromatography-tandem mass spectrometry assessment of docetaxel metabolism. Tissue culture medium samples were centrifuged at 12,000 × *g* for 6 minutes. Two hundred microliters of each resulting supernatant were added to a microcentrifuge tube and mixed with 10 μL of 10 μg/mL paclitaxel internal standard in methanol and 1 mL of acetonitrile. Each sample was vortexed for 1 minute on a Vortex Genie (Scientific Industries, Inc., Bohemia, NY) set at 8, and then centrifuged at 12,000 × *g* for 6 minutes. The resulting supernatant was pipetted into a 12 × 75 mm glass tube and evaporated to dryness under a stream of nitrogen. The dried residue was reconstituted in 200 μL of methanol/water/formic acid (50:50:0.1, v/v/v), and 100 μL were injected into the LC/MS/MS system. The 200 μL cytochrome *P*450 incubation mixtures were processed directly in a similar manner, except no internal standard was used. The dried residues were reconstituted in 100 μL of methanol/water (50:50, v/v) and 20 μL were injected into the LC/MS/MS system.

For most hepatocyte incubation samples, the LC system consisted of a Waters 2695 Alliance system (Waters Corporation, Milford, MA) with a Phenomenex Luna C18(2) (4.6 × 100 mm, 5 μm) column (Phenomenex, Torrance, CA). The isocratic mobile phase, consisting of acetonitrile/water/formic acid (50:50:0.1, v/v/v), was pumped at 0.5 mL/min. For all cytochrome *P*450 incubation mixtures and several hepatocyte incubation samples, the LC system consisted of an Agilent 1100 autosampler and binary pump (Agilent Technologies, Palo Alto, CA), a Phenomenex SYNERGI Hydro RP column (2 × 100 mm, 5 μm),

Table 1. Donor information for human hepatocyte preparations

Donor	Age (y)	Sex	Race	Cause of death	Drug history	Viability (%)
HH1117	68	F	Caucasian	Cerebral vascular accident	Labetalol, verapamil, clonidine, metoclopramide, simvastatin, clopidogrel	82
HH1121	65	F	Caucasian	Cerebral vascular accident	No medications reported	78
HH1122	46	F	Caucasian	Head trauma	Triamterene, propranolol, levothyroxine, verapamil, alprazolam, modafinil, glatiramer acetate	76

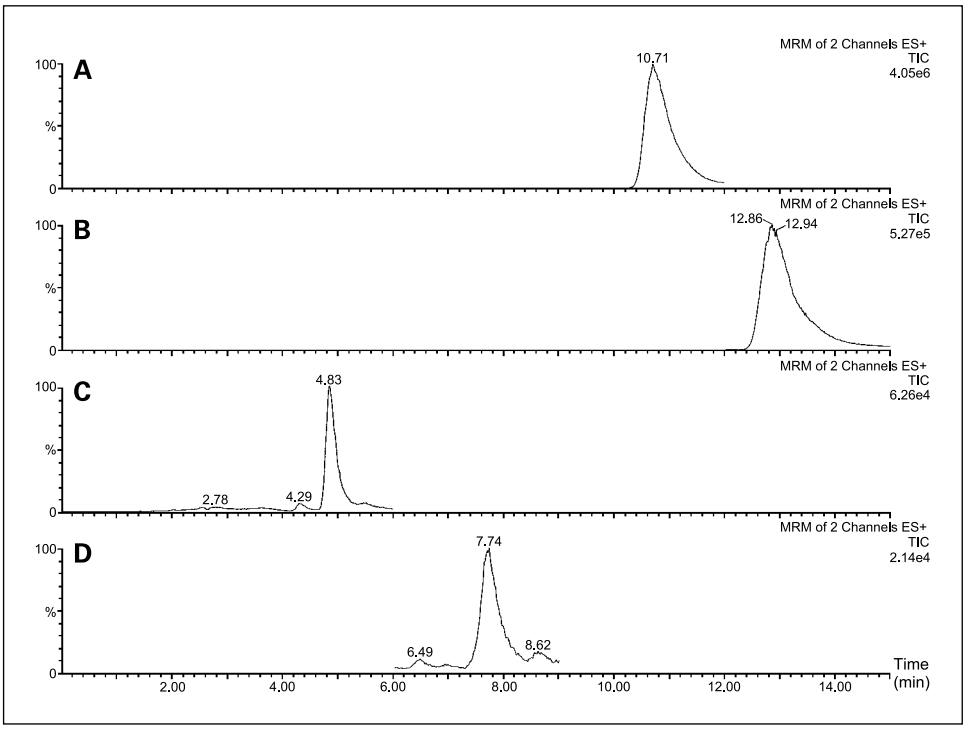

Fig. 1. LC/MS/MS chromatogram of total ion current of product ions monitored for docetaxel (*A*); paclitaxel internal standard (*B*); the *tert*-butyl – hydroxylated metabolite of docetaxel (*C*); and baccatin ring-hydroxylated metabolites of docetaxel (*D*).

and a mobile phase of acetonitrile/water/formic acid (37:63:0.1, v/v/v) that was pumped at 0.2 mL/min.

The mass spectrometer was a Micromass Quattro-micro bench-top, triple-stage mass spectrometer (Waters Corporation). The mass spectrometer operated under the following parameters: capillary voltage, 4.0 kV; cone voltage, 25.0 V; source and desolvation temperatures, 120°C and 400°C, respectively. The cone and desolvation gas flows were 110 and 550 L/h, respectively. Both the low mass and high mass resolution on quadrupole 1 and quadrupole 2 were set at 12. The collision cell was set at 35 V. The system was operated in electrospray ionization – positive mode with multiple reaction monitoring detection. The precursor > product ions monitored were the following: m/z 846 > 320 and m/z 846 > 549 (*tert*-butyl – hydroxylated metabolite); m/z 846 > 248 and m/z 846 > 565 (ring-hydroxylated metabolites); m/z 830 > 248 and m/z 830 > 549.5 (docetaxel); and m/z 876 > 308 and m/z 876 > 591 (paclitaxel internal standard). The internal standard ratio for each sample was calculated as the ratio of the total ion current of the two product ions monitored for docetaxel or metabolites divided by the total ion current of the two product ions monitored for paclitaxel. Standard curves of docetaxel were constructed by plotting the internal standard ratio versus the known concentration of docetaxel in prepared standards. Duplicate standard curves containing docetaxel concentrations of 1, 3, 10, 30, 100, 300, and 1,000 nmol/L were included with each analytic run. Standard curves were fit by linear regression with weighting by $1 / y^2$, followed by back-calculation of concentrations. The system used Mass Lynx software version 4.0 (Waters Corporation) for operation and data acquisition.

Under the LC/MS/MS conditions described for hepatocyte incubations, docetaxel eluted at ~10.7 minutes (Fig. 1A), and paclitaxel internal standard eluted at ~13 minutes (Fig. 1B). The assay had a

Table 2. Effects of rifampin and hyperforin on docetaxel metabolism by cultured human hepatocytes

Metabolite	Inducer	Hepatocyte preparation			
		HH 1117	HH 1121	HH 1122	*P**
Tert-butyl – hydroxylated	DMSO (0.1%)	1.00†	1.00	1.00	—
	Rifampin (10 μmol/L)	6.81	17.50	32.00	0.07
	Hyperforin (0.1 μmol/L)	1.33	2.25	2.00	0.045
	Hyperforin (0.5 μmol/L)	2.52	6.00	6.00	0.040
	Hyperforin (1.5 μmol/L)	2.63	—	7.00	0.166
Ring-hydroxylated	DMSO (0.1%)	1.00	1.00	1.00	—
	Rifampin (10 μmol/L)	9.00	18.5	23.25	0.03
	Hyperforin (0.1 μmol/L)	1.54	1.83	1.75	0.008
	Hyperforin (0.5 μmol/L)	3.31	4.5	4.00	0.007
	Hyperforin (1.5 μmol/L)	3.08	—	5.00	0.098

*Student's 1-sample, 1-sided *t* test compared with DMSO control.
† Values represent -fold increase in concentration of metabolite compared with the concentration in control hepatocyte cultures treated with DMSO.

lower limit of quantitation (38) of 1 nmol/L and was linear over the range of 1 to 1,000 nmol/L. Based on three triplicate, back-calibrated standard curves, the accuracy of the assay ranged between 93% and 106%. Based on 3-day assays of quality control samples containing 5, 50, or 500 nmol/L docetaxel (six at each concentration), the interday precision of the assay was 2% to 6% at any given concentration and the intraday precision of the assay was 2% to 10%. No materials in incubation medium from hepatocyte cultures interfered with the quantitation of docetaxel or internal standard. Because authentic standards of docetaxel metabolites were not available, absolute quantitation of docetaxel metabolites was not possible and, therefore, relative amounts of these materials present in incubation medium were expressed as the ratio of the area under the proposed metabolite peak to the area under the respective internal standard in that incubation medium. Under the LC/MS/MS conditions described for cytochrome *P*450 incubations, docetaxel eluted at ~28 minutes.

Statistics. Because metabolism in DMSO cultures was assigned a value of 1 and induction was expressed as fold induction over control, Student's one-sample, one-sided *t* test was used to test the null hypothesis that induction of docetaxel metabolism yielded a mean value >1. Spearman's correlation was used to assess the strength of dose dependency of induction of metabolism by hyperforin. All statistical analyses were done with SPSS software, version 12 (SPSS, Inc., Chicago, IL).

Results

As indicated in Table 2, rifampin induced metabolism of docetaxel between 6.8- and 32-fold over the metabolism of docetaxel observed in control hepatocyte cultures pretreated with DMSO alone. Hyperforin also induced metabolism of docetaxel and did so in a dose-dependent manner (Table 2). Of note, two sites of docetaxel metabolism were characterized by the LC/MS/MS assay used. Under the LC conditions used for analyzing hepatocyte incubations, the first of these metabolites eluted at ~4.8 minutes (Fig. 1C) and was the previously described *tert*-butyl–hydroxylated metabolite resulting from CYP3A4 metabolism of docetaxel (Fig. 2B; refs. 33, 39–42). This structural assignment was based upon the mass spectrum fragmentation pattern shown in Fig. 3B. Specifically, two products ions, with *m/z* 549 and 320, respectively, were observed (Figs. 2B and 3B). The product ion with *m/z* 549 represented the sodium adduct of the baccatin ring and was also observed in the MS fragmentation of docetaxel (Figs. 2A and 3A). The product ion with *m/z* 320 (Fig. 3B) represented the sodium adduct of a molecular fragment 16 atomic mass units greater than the *m/z* 304 fragment resulting from the sodium adduct of the side chain portion of docetaxel (Figs. 2A and 3A). Induction of this metabolism ranged between 1.3- and 2-fold for 0.1 μmol/L hyperforin to 2.6- to 7-fold for 1.5 μmol/L hyperforin (Spearman ρ, 0.82; $P = 0.01$; Table 2). Of note, an apparent metabolite of docetaxel, characterized by hydroxylation of the baccatin ring, was also observed in the hepatocyte incubations (Figs. 1D, 2C, and 3C). Under the LC conditions used for analyzing hepatocyte incubations, this proposed metabolite eluted at ~7.7 minutes (Fig. 1D). Structural assignment of ring hydroxylation was based on the mass spectrum fragmentation pattern shown in Fig. 3C. Specifically, two product ions, with *m/z* 565.5 and 304, respectively, were observed (Figs. 2C and 3C). The product ion with *m/z* 565.5 represented the sodium adduct of a fragment 16 atomic mass units greater than the *m/z* 549 ion observed in the MS fragmentation pattern of docetaxel (Figs. 2A and 3A). The product ion with *m/z* 304 represented the sodium adduct of the molecular fragment known to result from the side chain portion of docetaxel (Figs. 2A and 3A). This proposed baccatin ring hydroxylation of docetaxel was also induced by hyperforin in a dose-dependent manner, with induction ranging from 1.5- to 1.8-fold for 0.1 μmol/L hyperforin to 3- to 5-fold for 1.5 μmol/L hyperforin (Spearman ρ, 0.76, $P = 0.03$; Table 2).

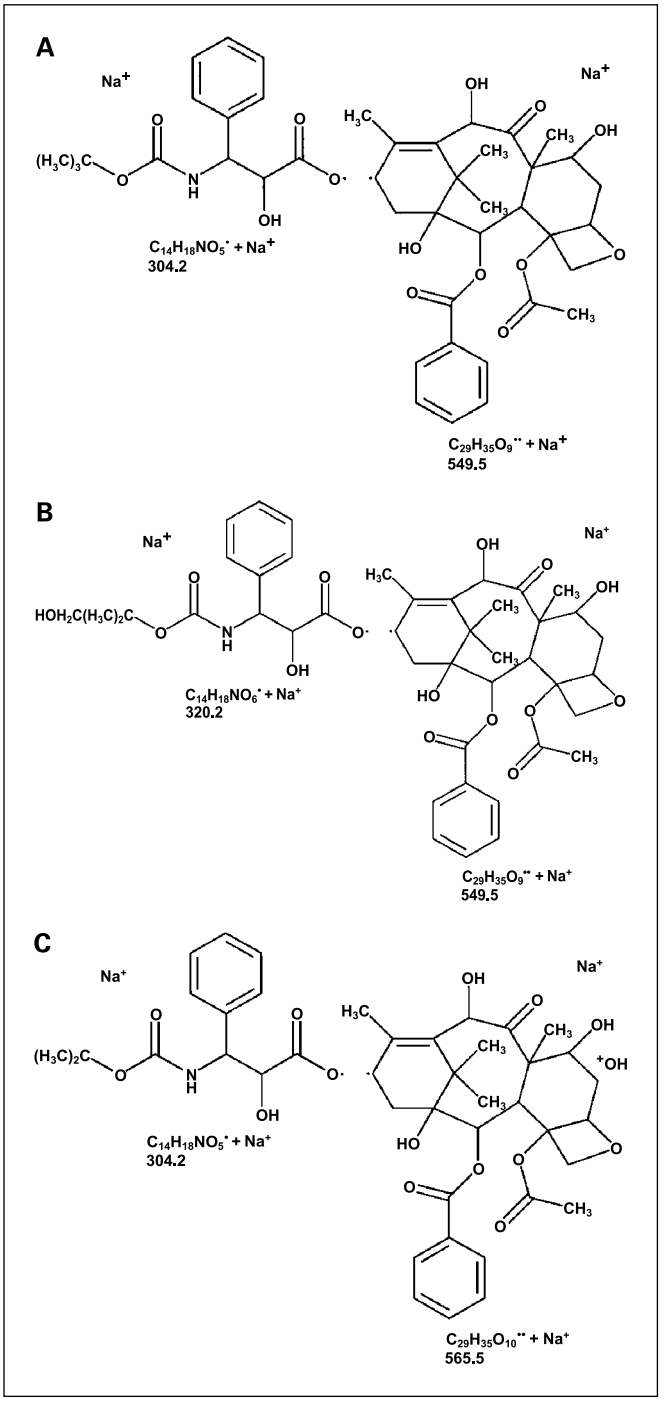

Fig. 2. Structures of ions produced by fragmentation of docetaxel (*A*); the *tert*-butyl – hydroxylated docetaxel metabolite eluting at 4.8 minutes (*B*); and the proposed baccatin ring-hydroxylated docetaxel metabolite eluting at 7.74 minutes (*C*).

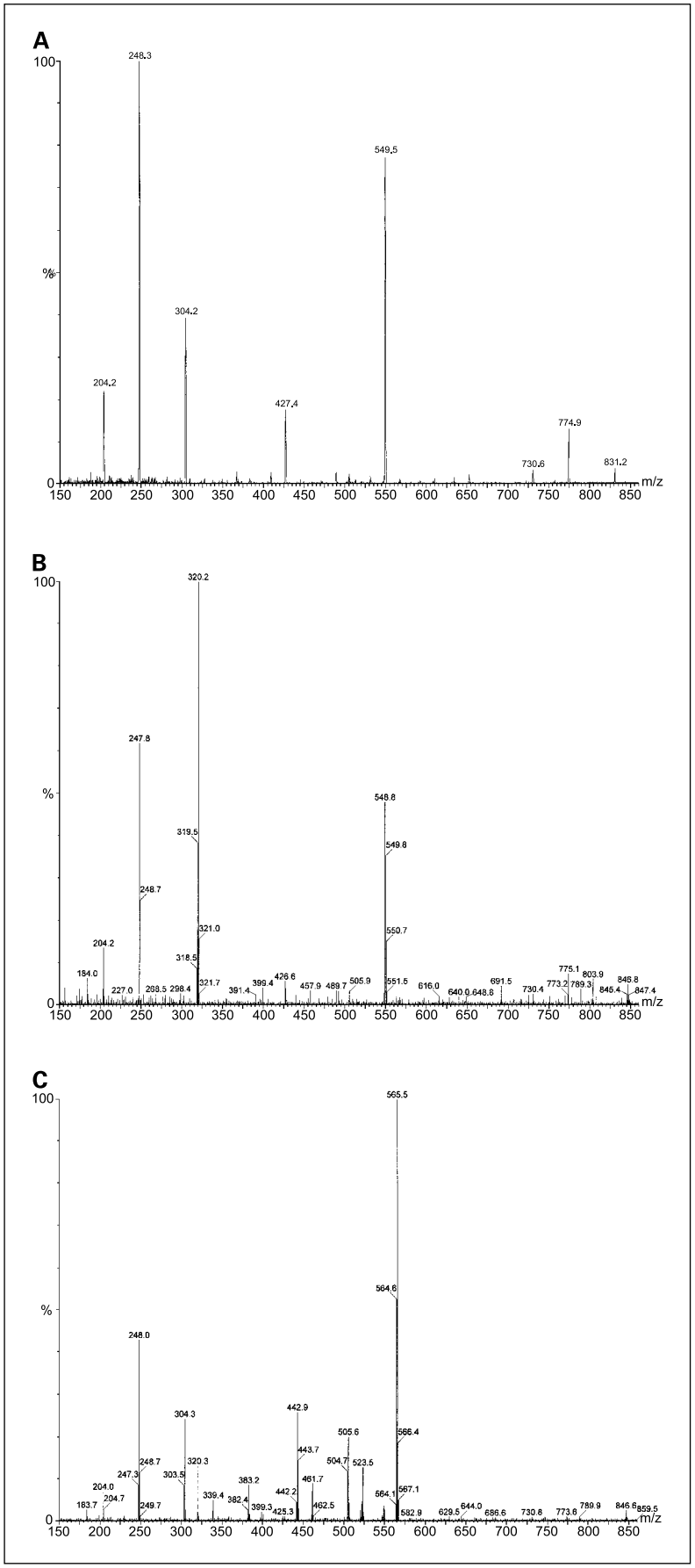

Fig. 3. LC/MS/MS spectra of the sodium adduct of docetaxel (*A*); the sodium adduct of the *tert*-butyl – hydroxylated docetaxel metabolite eluting at 4.8 minutes (*B*); and the sodium adduct of the proposed baccatin ring-hydroxylated docetaxel metabolite eluting at 7.74 minutes (*C*).

Incubation of docetaxel with cloned, human CYP3A4 confirmed that isoform as being responsible for production of the *tert*-butyl–hydroxylated metabolite that eluted at 4.8 minutes under LC conditions initially used to analyze hepatocyte incubations and which eluted at ~8.4 minutes under the LC conditions used to analyze cytochrome *P*450 incubation mixtures (Fig. 4A and C). Under the LC conditions used to analyze cytochrome *P*450 incubations, the proposed ring-hydroxylated metabolite that eluted at ~7.7 minutes under the LC conditions initially used to analyze hepatocyte incubations was resolved into two peaks with retention times of ~13.5 and 16.3 minutes, respectively (Fig. 4B). Both of these metabolites were produced by cloned, human CYP3A4 (Fig. 4D), whereas only the metabolite eluting at 16.3 minutes was produced by cloned, human CYP2C8 (Fig. 4E). No metabolites were observed when docetaxel was incubated with CYP2C9.

Discussion

Primary cultures of human hepatocytes offer a number of advantages over other systems commonly used in drug metabolism research. Hepatocytes are intact systems containing all of the necessary cofactors for the oxidative, reductive, and conjugative metabolism of xenobiotics. Yet, their simplicity compared with liver slices, whole perfused organs, or *in vivo* animal systems allows for the elimination of confounding factors such as blood flow and blood protein binding.

In this study, we have shown that human hepatocytes exposed chronically to the St. John's wort constituent hyperforin (up to 1.5 μmol/L) had increased cytochrome *P*450 activity as evidenced by induction of docetaxel metabolism. The limit of 1.5 μmol/L hyperforin was due to the fact that chronic (48 hours) exposure of human hepatocytes to hyperforin concentrations >1.5 μmol/L has been shown to be cytotoxic (13). However, the induction observed with 0.1 and 0.5 μmol/L hyperforin is more likely to reflect the potential clinical situation because peak hyperforin concentrations in plasma of patients taking the commonly used St. John's wort extract dose of 300 mg tid are ~0.28 μmol/L and steady-state concentrations are ~0.18 μmol/L (43). Although docetaxel metabolism was increased in each of the three hepatocyte preparations, there was some variability in the degree of induction at each of the hyperforin concentrations used. This might reflect a number of factors including age, sex, and medications of the donor. In our studies, each hepatocyte donor was female (44). Our data are unlikely to reflect drugs used by the hepatocyte donors or during hepatocyte preparation because studies were carried out on day 5 or 6 of culture, and our experience is that the effects of drugs used by donors do not last this long. Our use of 100 μmol/L docetaxel reflected several considerations. Whereas 100 μmol/L docetaxel is well above the concentrations achieved in plasma of patients receiving docetaxel, it is within the range of concentrations achieved in the livers of mice treated with i.v. docetaxel in antitumor efficacy studies (45). Furthermore, whereas 100 μmol/L is above the estimated 1 to 10 μmol/L K_m of CYP3A for docetaxel, our studies were done in a setting of presumed induction with increased enzyme content and activity. Therefore, we chose to use 100 μmol/L docetaxel to guarantee the saturating docetaxel concentrations required to perform suitable assessments of enzyme induction. Overall, our data are consistent with reports that St. John's wort administration induces metabolism and MDR1 P-glycoprotein transport of other CYP3A4 substrates, such as cyclosporine, indinavir, irinotecan, and imatinib, resulting in decreased plasma or blood concentrations of these drugs (refs. 8–11, 46, 47).

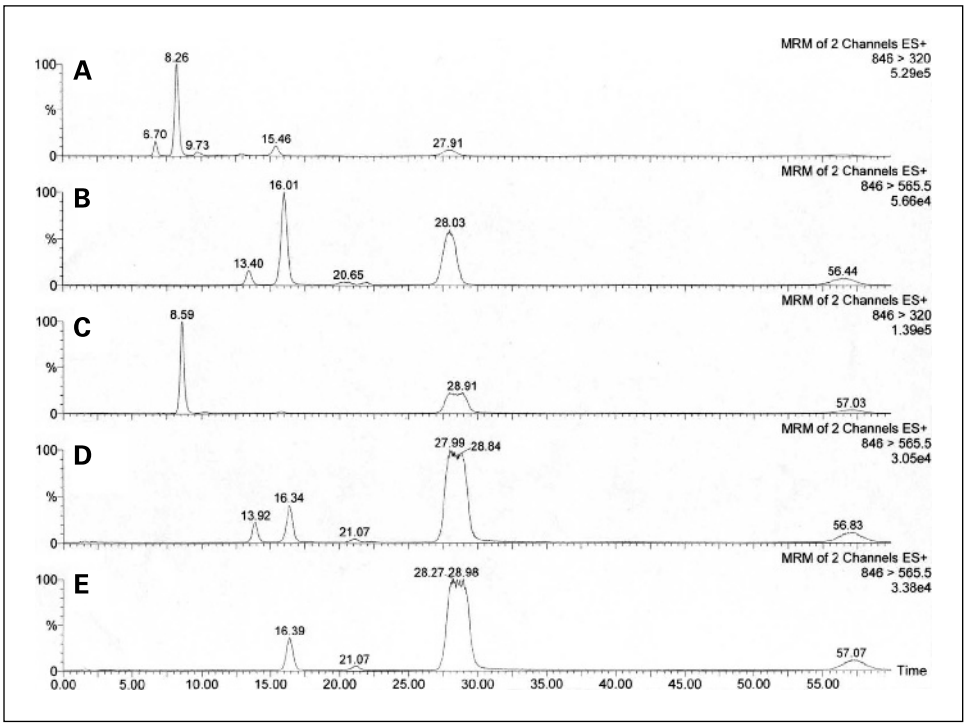

Fig. 4. LC/MS/MS chromatograms of total ion current of product ions monitored for the *tert*-butyl – hydroxylated metabolite produced by incubating docetaxel with human hepatocytes (*A*); the baccatin ring-hydroxylated metabolites produced by incubating docetaxel with human hepatocytes (*B*); the *tert*-butyl – hydroxylated metabolite produced by incubating docetaxel with cloned, human CYP3A4 (*C*); the baccatin ring-hydroxylated metabolites produced by incubating docetaxel with cloned, human CYP3A4 (*D*); and the baccatin ring-hydroxylated metabolite produced by incubating docetaxel with cloned, human CYP2C8 (*E*).

It should be noted that whereas ring-hydroxylated metabolites of docetaxel and CYP2C8 metabolism of docetaxel have not been reported previously, these observations might reflect the high concentrations of docetaxel used in our studies. Whereas our data indicate hydroxylation of the baccatin ring of docetaxel is catalyzed by both CYP3A4 and CYP2C8, the exact positions hydroxylated remain to be elucidated. It is possible that one of the ring-hydroxylated metabolites is analogous to the 6-α-hydroxy metabolite of paclitaxel produced by CYP2C8.

Although the data presented here clearly document the ability of hyperforin to induce the *in vitro* metabolism of docetaxel by human hepatocytes, *in vivo* preclinical and, eventually, clinical studies will be required to document the overall impact of hyperforin on docetaxel clearance. However, ethical concerns could make design of a clinical study involving exposure of patients to the intentional combination of hyperforin and docetaxel difficult. If an inactivating outcome is expected, the only way in which clinical studies could be done would be in a cohort of patients already taking St. John's wort. However, using each patient as his or her own control would require studying the pharmacokinetics of docetaxel in that patient when that patient would not be taking St. John's wort. If the clearance of St. John's wort would be decreased while the patient were off St. John's wort and the patient experienced an increased area under the curve, the patient might well experience increased neutropenia or other toxicity, again raising serious ethical issues about the ability to do such a trial. Nevertheless, in that many antineoplastic agents are substrates for CYP3A and other cytochrome *P*450s induced by St. John's wort, the chronic use of St. John's wort by patients with cancer could result in subtherapeutic exposure to those antineoplastic agents.

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