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SHORT COMMUNICATIONS

Taxol metabolism in rat hepatocytes

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Abstract—Metabolism of the anticancer drug taxol was investigated in freshly isolated rat hepatocytes. Two main metabolites were separated by reversed-phase HPLC and shown by tandem mass spectrometry to be monohydroxylated metabolites. Kinetic studies revealed apparent K_m values of 68 and 61 μM with identical V_{max} values for the two metabolites. Verapamil and midazolam, but not phenacetin, showed concentration-dependent inhibition of taxol metabolism with both metabolites being affected equally. The IC_{50} was about 100 μM for verapamil and 25 μM for midazolam. These observations demonstrate for the first time *in vitro* metabolism of taxol and suggest that the metabolism may be subject to potentially important interactions with numerous other drugs.

Taxol, a natural product isolated from the bark of the Western yew tree, is a potent inhibitor of cell replication that blocks cells in the mitotic phase of the cell cycle [1]. It has shown promising activity against a variety of human malignancies, including breast, ovarian and lung tumors as well as acute leukemias* [2]. In spite of rapidly widening clinical use, little information is available on the biological fate of taxol. As only small amounts of unchanged drug are found in the urine of both humans [2] and animals [3], it would appear that taxol is cleared mainly by metabolism. This has been demonstrated in the rat, where multiple metabolites are formed, with two oxidative metabolites predominating [3]. These metabolites were all excreted in the bile; no metabolites were detected in urine.

Because of the difficulties involved in studies of drug metabolism from biliary excretion, in particular quantitative and kinetic studies, it may be an advantage to use an *in vitro* metabolic system. In the present study we have explored the utility of the rat hepatocyte suspension for the study of taxol metabolism.

Materials and Methods

Materials. Taxol was purchased from Calbiochem (San Diego, CA), and [^{3}H]taxol (15–30 Ci/mmol, generally labeled) from Moravek Biochemicals (Brea, CA). (±)Verapamil and phenacetin were from the Sigma Chemical Co. (St. Louis, MO), and midazolam HCl was from Hoffmann-La Roche Inc. (Nutley, NJ). Other chemicals were from standard sources.

Incubates. Hepatocytes from male Long Evans rats (200–250 g) were isolated by collagenase perfusion [4] as modified by McMillan and Jollow [5]. Cell viability was > 80%. Washed hepatocytes were suspended in 2 mM hydroxyethylpiperazine-ethanesulfonic acid/phosphate-buffered saline (HEPES/PBS)† with dextrose (11 mM), $CaCl_2$ (2 mM), $MgSO_4$ (0.6 mM) and $NaHCO_3$ to pH 7.4. Aliquots (0.5 mL with $3.7 \pm 0.2 \times 10^6$ [mean ± SD] viable cells) were incubated with 3–300 μM taxol and 0.5 μCi of [^{3}H]-taxol at 37° for up to 3 hr with gentle rocking.

Taxol metabolite assay. Taxol and its metabolites were extracted with 5 mL of ethyl acetate. The ethyl acetate extract was evaporated to dryness at room temperature and reconstituted in 400 μL of mobile phase used in the reversed-phase HPLC analysis. One 100-μL aliquot was counted directly by liquid scintillation spectrometry. The recovery of taxol and metabolites was about 90%. Another aliquot was subjected to reversed-phase HPLC analysis using a Curosil-G 6 μm (250 × 3.2 mm i.d.) column (Phenomenex, Torrance, CA) with 35% acetonitrile in water as mobile phase and 229 nm UV detection. The flow rate was 0.6 mL/min. Eluate fractions (1 mL) were collected for counting.

Identification of metabolites by mass spectrometry. Data were obtained through the Medical University of South Carolina Mass Spectrometry Research Resource Facility using an HX110/HX110 tandem mass spectrometer (JEOL, Tokyo). Liquid secondary ion mass spectrometry (LSIMS) ionization was done by bombardment of the sample in glycerol with 9 keV cesium ions. Mass spectra were acquired by scanning MS-I at *m/z* 10-1000 with an accelerating voltage of 10 kV. MS/MS spectra used 7 keV collision-activated dissociation with helium as the collision gas. MS-II was operated in the B/E linked scan mode, scanning *m/z* 0-900.

Results and Discussion

After incubation of the hepatocytes with [^{3}H]taxol, the reaction mixtures were extracted with ethyl acetate and,

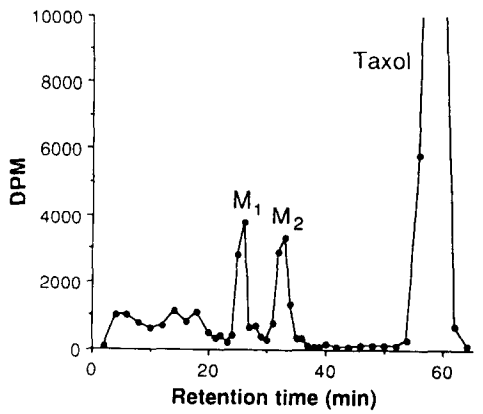

Fig. 1. Reversed-phase HPLC of ethyl acetate extract of rat hepatocyte incubate with 30 μM taxol. One-minute fractions were collected, and the radioactivity was measured by liquid scintillation spectrometry.

* Arbuck SG, Current status of the clinical development of taxol. Second National Cancer Institute Workshop on Taxol and Taxus, Alexandria, VA, September 23–24, 1992.

† Abbreviations: HEPES, hydroxyethylpiperazine-ethanesulfonic acid; PBS, phosphate-buffered saline; and LSIMS, liquid secondary ion mass spectrometry.

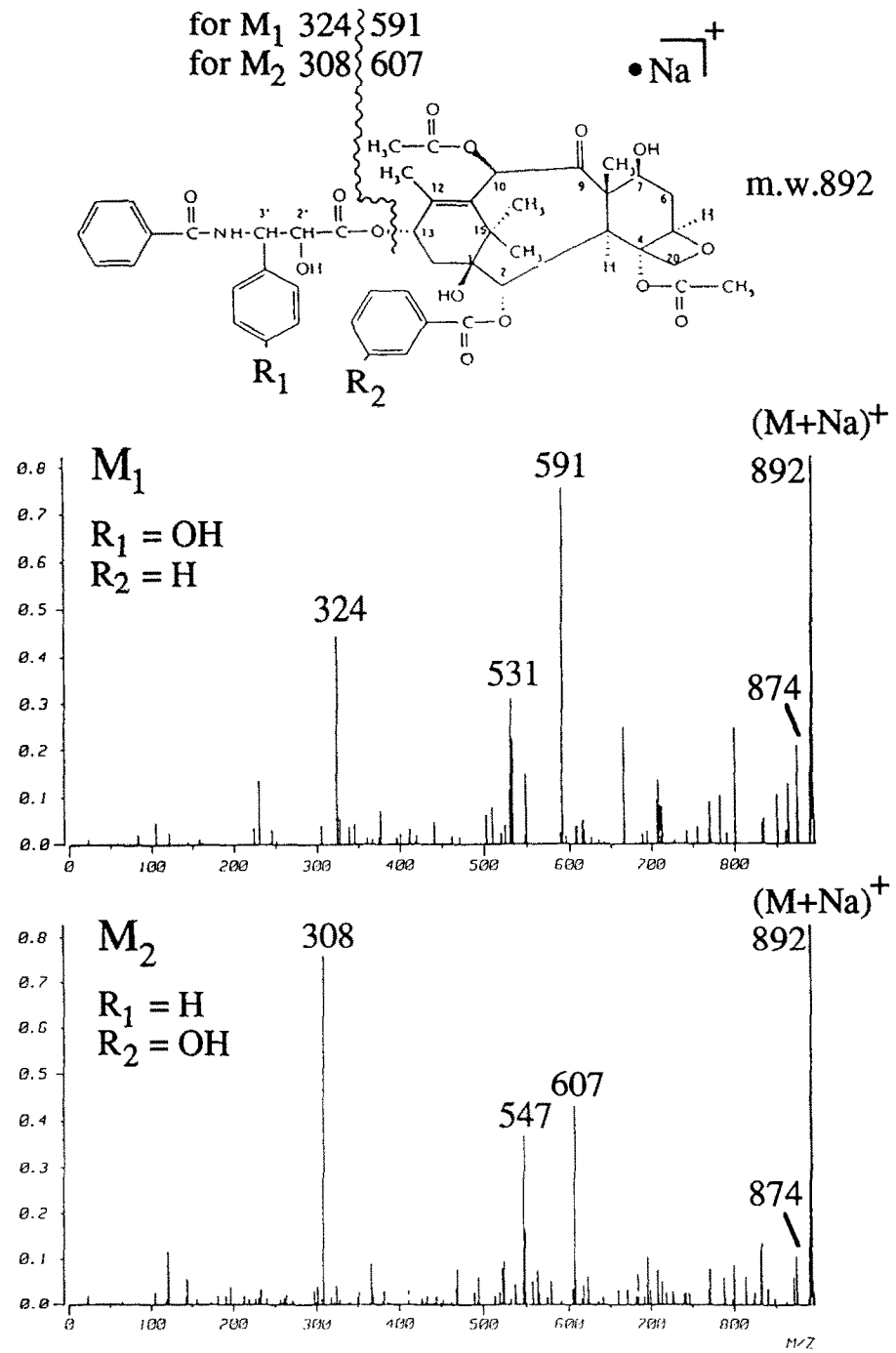

Fig. 2. Tandem mass spectrometry of metabolites M_1 and M_2. The metabolites were isolated by HPLC as in Fig. 1, and the mobile phase was removed by lyophilization. The ordinate represents relative intensity, expressed in arbitrary units.

following evaporation of the extracts and reconstitution in mobile phase, subjected to reversed-phase HPLC. Fractions of the column effluent were collected and radioactive components detected by liquid scintillation spectrometry. A typical chromatogram is shown in Fig. 1, depicting two metabolites, M_1 and M_2, in addition to unchanged taxol. For structure identification the regular 0.5-mL incubate was scaled up to 16 mL (118×10^6 cells), and the metabolites were isolated as in Fig. 1. After lyophilization, each of the metabolites (20–30 μg) was analyzed by tandem mass spectrometry. Both M_1 and M_2 had identical $(M + Na)^+$ ions of m/z 892 (Fig. 2) in addition to highly characteristic MS fragmentation, including a very prominent cleavage at the C13 position [3, 6]. M_1 had a side-chain fragment of

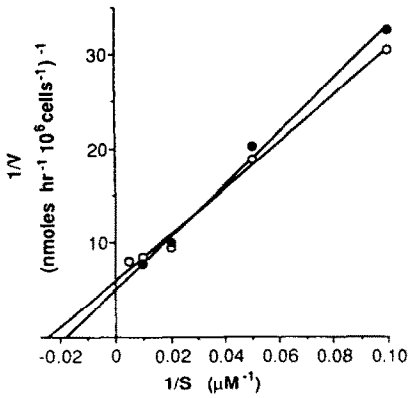

Fig. 3. Lineweaver–Burk plots of velocity of formation of metabolites M_1 (●) and M_2 (○) vs taxol concentration in one experiment.

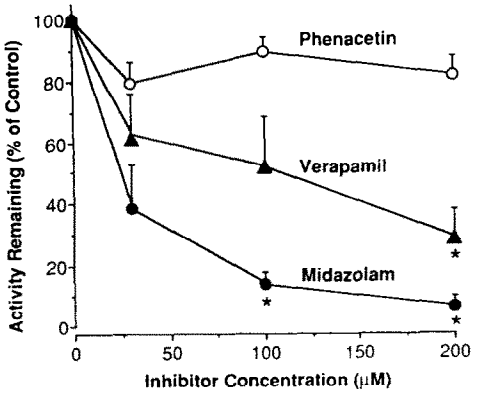

Fig. 4. Effects of verapamil, midazolam and phenacetin on the formation of taxol metabolites. As both metabolites were affected equally, the figure shows the sum of M_1 and M_2 (means ± SEM, N = 3). The taxol concentration used was 50 μM. The control activity was 0.062 nmol·hr^{-1} (10^6 cells)$^{-1}$. Key: (*) Significantly different from control ($P < 0.02$; paired t-test).

m/z 324 as compared with m/z 308 for unchanged taxol, indicating hydroxylation of this portion of the molecule, with a taxane ring fragment of m/z 591, i.e. identical to unchanged taxol. M_2, on the other hand, had an unchanged side-chain fragment of m/z 308 but with a taxane ring fragment of m/z 607, indicating hydroxylation of this portion of the taxol molecule (Fig. 2). These observations are identical to the findings made by Monsarrat *et al.* [3] in the intact rat.

The radioactivity eluting at 4–20 min in Fig. 1 appeared to be due mostly to minor impurities in the labeled taxol, as it was also present in extracts after 0 min incubations. However, the two minor peaks at 14 and 18 min are most likely taxol metabolites, too small for structure identification. Minor metabolites at these retention times were also present in rat bile extracts [3]. As in the intact rat, there was no evidence of glucuronic acid or sulfate conjugates of taxol metabolites, as determined by incubations with β-glucuronidase and sulfatase. HPLC analysis of the aqueous phase after ethyl acetate extraction did not reveal any radioactive peaks in addition to those shown in Fig. 1. Although taxol contains several ester linkages, potentially prone to hydrolytic cleavage by esterases, no such metabolites were found in this study.

Our study used rat hepatocytes as the most physiological model of *in vivo* metabolism, allowing us to detect potential glucuronidation and sulfation reactions. As no conjugation was detected, hepatic microsomes may also be a useful model for taxol metabolism. Indeed, in preliminary experiments both M_1 and M_2 were formed in about equal amounts using a rat liver microsomal preparation with an NADPH-generating system.

In kinetic studies with the hepatocyte preparation and taxol concentrations ranging from 10 to 300 μM, the metabolism was linear for at least 3 hr and saturable. The double-reciprocal plots of velocity vs taxol concentration were linear for both M_1 and M_2, as shown for a typical experiment in Fig. 3. The apparent K_m values were 68 ± 23 μM (mean ± SD) for M_1 and 61 ± 7 μM for M_2 (N = 3). The corresponding apparent V_{max} values were 0.19 ± 0.07 and 0.19 ± 0.05 nmol·hr^{-1}·(10^6 cells)$^{-1}$ for M_1 and M_2, respectively. Thus, the kinetics of formation of the metabolites were identical.

In further experiments the influence of cytochrome P450 isoenzyme-selective substrates on taxol metabolism was tested. We used an approach adopted from a study of the metabolism of propafenone [7] with verapamil being a substrate for P450 3A and P450 1A2, midazolam a substrate for P450 3A, and phenacetin a substrate for P450 1A2. As shown in Fig. 4, both verapamil and midazolam (30–200 μM) were inhibitors of taxol metabolism. The IC_{50} was about 100 μM for verapamil and 25 μM for midazolam. Of particular interest was the fact that both M_1 and M_2 were inhibited to an identical extent. In contrast, phenacetin (30–200 μM) had no effect on taxol metabolism.

In conclusion, our experiments demonstrate that isolated hepatocytes are a useful model system for studies of the metabolism of taxol. The same two major metabolites are formed *in vitro* as *in vivo*, and they are formed in amounts readily identified by mass spectrometric techniques. Further reactions did not occur to any significant extent, including potential conjugation. The apparent enzyme kinetics could easily be established, demonstrating identical behavior of the two metabolites. Inhibition studies suggest that the oxidative attack leading to both metabolites may be mediated by P450 3A enzyme(s). If also observed in humans, potential interactions by numerous anticancer [8] and other [9] drugs as well as dietary constituents [10] could result in decreased clearance of taxol during therapy.

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