Notes

Isolation, Structural Determination, and Biological Activity of 6α-Hydroxytaxol, the Principal Human Metabolite of Taxol†,⊥

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The principal biotransformation product of taxol was found to be identical for human hepatic microsomes, human liver slices, and patient bile samples. We have isolated this metabolite from the bile of a patient given taxol, and we report its structure and its cytotoxicity relative to taxol. The NMR and SIMS data presented here indicate that, in humans, taxol is regiospecifically hydroxylated at the 6-position on the taxane ring and that this hydroxyl is stereospecifically placed *trans* to the hydroxyl at position 7, yielding 6α-hydroxytaxol. This metabolite is apparently not formed in rats. Tests of the growth inhibition potential of 6α-hydroxytaxol versus taxol in two human tumor cell lines showed that the metabolite was approximately 30-fold less cytotoxic than taxol. Thus the cytochrome P-450-mediated biotransformation of taxol to 6α-hydroxytaxol can be classified as a detoxification reaction.

Introduction

Taxol is the earliest example in a class of diterpenoid antineoplastic agents that have a unique mechanism of action associated with cellular microtubule formation. In contrast to vinca alkaloids, taxol increases polymerization of microtubules, stabilizes them once polymerized, and slows depolymerization.[1,2] These actions result in the blockade of mitosis in the late G2 phase of the cell cycle. Taxol is a natural product that was extracted from the bark of the Pacific yew tree, but other sources are under intense study or development. A number of clinical trials that use taxol are in progress, and it appears that taxol is efficacious against several tumor types.[3] Taxol was found to be metabolized by cytochrome P-450 enzymes,[4] but it was unclear whether biotransformation reactions would affect the efficacy of taxol in humans.

When taxol is given to patients as a 24-h intravenous infusion, metabolites are not usually found in plasma,[5] presumably because metabolites are preferentially sequestered to bile. Protocols that use a 3-h infusion time are becoming more common, however, and taxol metabolites have been detected in plasma under these conditions.[6] The metabolism of taxol in rats has been reported,[7] and several metabolites were isolated from rat bile and their structures were determined. Monsarrat *et al.* have identified 6-hydroxytaxol as a major human metabolite of taxol,[8] but details of their analysis have not been published. Because *de novo* syntheses of taxol and taxol metabolites are difficult, our objectives in these studies were to isolate a pure sample of the major taxol metabolite from human bile, to completely characterize its structure, and to study its biological activity.

Results and Discussion

The chemical structures of taxol (R = H) and 6α-hydroxytaxol (R = OH) are given in Figure 1. Proton NMR assignments for 6α-hydroxytaxol were obtained from the 1D, DQCOSY, and TOCSY spectra of the sample. The spectrum from the 1D experiment was used to obtain an approximate proton count by integration of each apparent resonance. A TOCSY experiment was conducted in order to group these resonances into spin-coupled sets; this approach was particularly useful in unraveling the numerous overlapping resonances of the aromatic protons. A DQCOSY experiment was then used to arrange the atoms that had been assigned into groups by the TOCSY experiment into the correct spatial order. Because of spectral overlap of the proton resonances for H6 and H3, a 1D homonuclear proton–proton decoupling experiment was necessary to reveal the H6 coupling pattern: H2 was irradiated to collapse the multiplet of H3 to a singlet. The sample warming that resulted from this decoupling experiment apparently hastened chemical exchange of the solute with the HOD in the solution. The hydroxyl proton at C6 was exchanged, thus simplifying the multiplet of H6 to a sharp doublet of doublets and allowing the stereochemistry of substituents at C6 to be determined (see below). The assignments for proton chemical shifts, resonance multiplicities, and coupling constants were calculated and sorted into tabular form using an in-house

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† The United States Adopted Name Council has given the name "paclitaxel" to refer to taxol and TAXOL. Abbreviations: 1D, one-dimensional; 2D, two-dimensional; DQCOSY, proton double quantum filtered correlation spectroscopy; TOCSY, proton total correlation spectroscopy; HMQC, proton–carbon heteronuclear multiple quantum coherence; HMBC, proton–carbon heteronuclear multiple bond connectivity; ROESY, rotating frame nuclear Overhauser spectroscopy; NOE, nuclear Overhauser effect; SIMS, secondary ion mass spectrometry.
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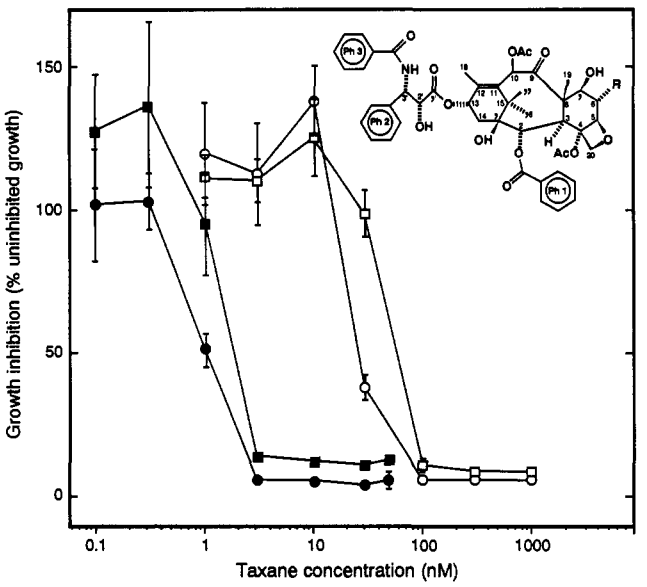

Figure 1. Inhibition of cellular growth by taxol (filled symbols; R = H) or by 6α-hydroxytaxol (open symbols; R = OH). Human-derived MOLT-4 (squares) or U-937 (circles) cells were exposed to taxanes for 72 h. The values reported are the average (± standard deviation) of drug exposure experiments conducted in triplicate.

computer program ZFD.[9] All resonances found in the ^1H NMR spectrum of the 6α-hydroxytaxol sample were assigned. The proton assignments made for 6α-hydroxytaxol are largely consistent with those for taxol,[10,11] except that the chemical shift for H6 was moved downfield by 2.07 ppm, consonant with the magnitude and direction of change expected for conversion from CH_2 to CHOH. Moreover only one H6 resonance was found, as indicated by a decrease of one nonexchangeable proton in the total proton count of 6α-hydroxytaxol when compared to the proton count of taxol and by the disappearance of geminal proton–proton coupling (14.8 Hz in taxol[10]) from the remaining H6 resonance.

After completing the proton assignments for the taxol metabolite preparation, 2D HMQC and HMBC experiments were used to assign the ^{13}C NMR spectra for protonated and quaternary carbons, respectively. Assignments for ^{13}C atoms, along with HMBC and HMQC correlations, were sorted into tabular form. Carbon chemical shift differences between 6α-hydroxytaxol and taxol were tabulated, and these data show that important differences between the two molecules exist at carbon atoms 5, 6, and 7. We interpret these data to indicate that the large, downfield movement of the C6 resonance ($\Delta\delta$ = 40.6 ppm) in 6α-hydroxytaxol versus taxol denotes amajor structural change at C6, that the magnitude and direction of this change are consistent with oxygen insertion at C6, and that the smaller changes at carbon atoms 5 and 7 are due to proximity. Moreover, the HMQC experiment showed only one crosspeak for C6 of 6α-hydroxytaxol, and the chemical shift of this crosspeak on the ^1H axis was consistent with the H6 proton assignment.

The NMR data indicated that the human metabolism of taxol to 6-hydroxytaxol is stereospecific. In order to determine the stereochemical result of this P-450-catalyzed hydroxylation reaction, energy-minimized structures for taxol, 6α-hydroxytaxol, and 6β-hydroxytaxol were calculated, and a transverse ROESY experiment was conducted on the unknown. The dihedral angles between H5 and H6 or between H6 and H7 derived from the energy-minimized structures of taxol, 6α-hydroxytaxol, and 6β-hydroxytaxol are shown in Figure 2, along with the experimentally determined vicinal proton–proton coupling values for taxol and its metabolite. Coupling constants found for the metabolite were $^3J_{5,6}$ = 1.4 Hz and $^3J_{6,7}$ = 8.8 Hz, and these values are consistent with the α-isomer of 6-hydroxytaxol because of their similarity to those for $H6_\beta$ of taxol (Figure 2). The decrease in J values of 11.0 to 8.8 Hz and of 2.3 to 1.4 Hz upon substitution of hydroxyl for hydrogen is reasonable because vicinal proton–proton coupling decreases with increased substituent electronegativity.[12,13] If effects of electronegativity were ignored, one might envision a change in $^3J_{6,7}$ from 6.7 to 8.8 Hz to be reasonable (hence lending credence to the β-isomer of 6-hydroxytaxol), but the accompanying change of $^3J_{5,6}$ from 9.7 to 1.4 Hz is unreasonable (Figure 2). A transverse ROESY experiment was conducted to confirm our interpretation of these results. A strong NOE crosspeak was found to exist between H6 and the methyl protons of C19 (named Me19 here); a similarly strong crosspeak was observed for taxol[14] between $H6_\beta$ and Me19 (NOE calculated distance of 2.39 Å) but not between $H6_\alpha$ and Me19. These data are consistent with a $H6_\beta$–Me19 distance of 2.42 Å derived from the energy-minimized model of 6α-hydroxytaxol, and they show that the 6-hydroxytaxol preparation reported here is the α-isomer.

Positive- and negative-ion SIMS analyses of taxol and its metabolite were conducted, with the negative-ion experiments generally giving stronger taxane-related signals (values reported are from negative-ion experiments). Similar analyses were conducted with baccatin III, a taxol analog that is devoid of the C13 side chain. The 6α-hydroxytaxol preparation showed a molecular ion of m/z 868 and a prominent 3-nitrobenzyl alcohol adduct ion of m/z 1022, whereas taxol showed ions at m/z 852 and 1006, respectively. Cleavage of the ester bond at C13 was facile under the conditions used. Fragment or molecular ions representing the taxane ring of taxol, baccatin III, or 6α-hydroxytaxol were found at m/z 525, 525, or 541, respectively. Ions at m/z 284, consistent with the C13 side-chain fragment, were found for taxol and for 6α-hydroxytaxol, but not for baccatin III. These data support the NMR structural arguments given above.

The cytotoxic potential of taxol and of 6α-hydroxytaxol were directly compared by exposing the human-derived cell lines MOLT-4 and U-937 to these taxanes for a period of 72 h. The pharmacological activity (IC_{50}) of taxol in the cell lines MOLT-4 and U-937 was 1.6 and 1.0 nM, respectively, whereas the values for 6α-hydroxytaxol were 53 and 29 nM in the same cell lines. The growth inhibition curves for these experiments are shown in Figure 1. The results given above were reproduced, within experimental error, in a separate series of experiments. These results indicate that 6α-hydroxytaxol is approximately 30-fold less potent than taxol in the *in vitro* inhibition of cell growth, and they suggest that the same rank order of potency might be operative *in vivo*.

Conclusions

The data presented here indicate that, in humans, taxol is regiospecifically hydroxylated at the 6-position on the taxane ring and that this hydroxyl is stereospecifically placed *trans* to the hydroxyl at position 7. Although other human taxol metabolites are known,[4,8] 6α-hydroxytaxol formation appears to be the principal taxol biotransfor-

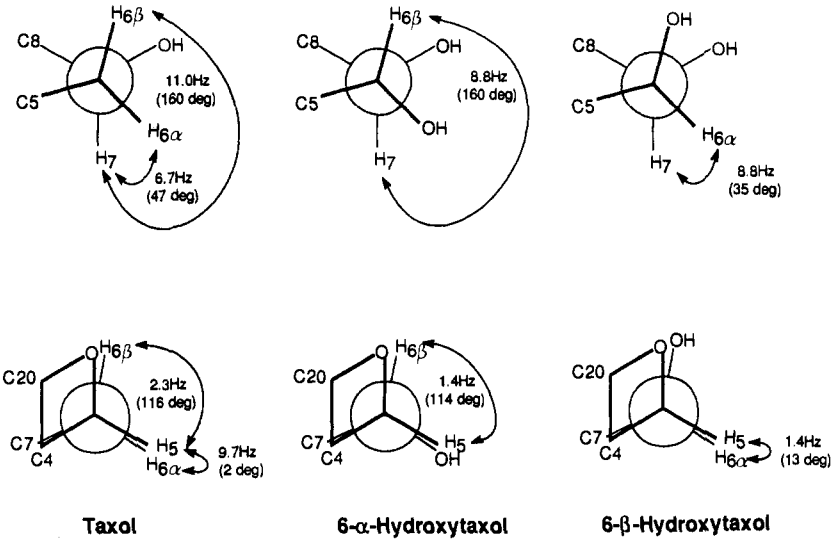

Figure 2. Newman projection of substituent dihedral angles, derived from energy-minimized structures for taxol, 6α-hydroxytaxol, and 6β-hydroxytaxol, (a) between C6 and C7 or (b) between C5 and C6. Experimentally determined vicinal proton–proton coupling values for taxol and its metabolite are shown.

mation reaction of humans, as judged from work with human hepatic microsomes, human liver slices, and patient bile samples.[4,15]

Few data exist on the comparative biological fate of taxol in mammals, but 6α-hydroxytaxol is apparently not a product of taxol metabolism in rats.[7] Moreover, analysis of the available data on identified rat metabolites[7,8] or minor, unidentified metabolites[16] yields no evidence that rats modify the taxane ring of taxol. Hence rats might not be an appropriate animal model for taxol pharmacology studies.

It appears that biotransformation does affect the efficacy of taxol in humans. 6α-Hydroxytaxol is less cytotoxic than taxol (Figure 1), a finding that is consistent with Kingston's generalizations[17] that modification of the taxane-ring skeleton reduces biological activity.

Experimental Section

Metabolite Isolation. Bile was collected from a patient who received taxol as a 24-h infusion as part of Cancer and Leukemia Group B protocol no. 9264 and was used in all experiments described here. This patient had prior insertion of a biliary drainage tube for management of underlying disease.

Only one taxol metabolite, shown here to be 6α-hydroxytaxol, was readily detected with the chromatographic system outlined below; this protocol does resolve two minor taxol metabolites formed by human hepatic microsomes and by liver slices.[4] Purification of 6α-hydroxytaxol involved an initial liquid–liquid extraction: 4 volumes of bile, 3 volumes of chloroform, and 10 volumes of 0.02 N NaOH were combined and were mixed vigorously. Emulsions that resulted from mixing were dispersed by centrifugation; after phase separation was complete, the aqueous layer was discarded. The organic layer was washed four times with 10 volumes of 0.02 N NaOH and once with 10 volumes of water. The colorless chloroform solution was evaporated to dryness, and the resulting residue was dissolved in 1:1 acetonitrile–water for further purification by analytical HPLC. HPLC conditions were as follows: injection volume, 100 μL; detection, 230 nm in UV; run time, 50 min; flow rate, 1.0 mL/min; gradient conditions (time in min:% water; remaining composition was acetonitrile), 0:90, 5:90, 30:35, 40:35. A Hewlett-Packard 1090 instrument was used and was equipped with an automatic switching valve and a Hewlett-Packard ODS Hypersil column (100 × 4.6 mm). Fractions eluting from 24.6 to 25.9 min were collected, pooled, and evaporated to dryness. Approximately 175 HPLC injections yielded 1.2 mg of residue.

The sample was judged to be pure by HPLC and by ^{1}H NMR and possessed an UV spectrum similar to that of taxol. Sufficient sample did not exist for routine elemental analysis, but additional evidence of sample purity may be assessed from its extinction coefficient. The extinction coefficient measured for the 6α-hydroxytaxol preparation was 26 200 $M^{-1}\cdot cm^{-1}$ at 230 nm (methanol); this value is comparable to the range of extinction coefficients reported for taxol by the Developmental Therapeutics Program, NCI (Bethesda, MD; 28 500–30 900 $M^{-1}\cdot cm^{-1}$).

Spectral Data. Proton (^{1}H; 499.843 MHz) and carbon (^{13}C; 125.697 MHz) NMR spectra were acquired at 27 °C using a Varian VXR-500S spectrometer equipped with a Sparc Station 2 workstation and a Nalorac (Martinez, CA) ID500-5 indirect detection 5-mm probe. ^{1}H chemical shifts are reported relative to TMS = 0 ppm and ^{13}C chemical shifts are reported relative to $CDCl_3$ = 77.0 ppm. Chemical shift referencing in indirect detection experiments was accomplished by using peak positions from the 1D spectra to assign chemical shifts to strong, sharp resonances in the 2D spectra. The entire dry metabolite residue noted above was dissolved in $CDCl_3$ and transferred to an NMR tube; the solvent and tube were previously shown to be free of impurities by NMR. Other methods for spectral acquisition, including those for measuring nuclear Overhauser effects, were identical to those previously described.[10] Comparative NMR data for taxol were from the report by Chmurny *et al.*[10,11]

Positive- and negative-ion SIMS were acquired using a Hewlett-Packard 5988 instrument equipped with a Phrasor cesium ion gun (Duarte, CA) operating at 10 keV. Samples (2–20 μg) of taxol, 6α-hydroxytaxol, or baccatin III (Developmental Therapeutics Program, NCI) were dissolved in a matrix mixture consisting of 12.5% 3-nitrobenzyl alcohol in glycerol (v/v).

Computer Simulation. Computer models were used to give simulated bond angles and NOE interactions for comparison with our experimental data. Energy-minimized structures for taxol, 6α-hydroxytaxol, and 6β-hydroxytaxol were calculated using INSIGHT II (version 2.1; Biosym Technologies, Inc., San Diego, CA). Parameters employed for simulation were those available with INSIGHT II and were not modified. Convergence was assumed when the calculation showed gradients <0.001 calorie along all coordinates.

Growth Inhibition Experiments. The cell lines MOLT-4 (derived from a human acute lymphoblastic leukemia[18]) and U-937 (derived from a human histiocytic lymphoma[19]) were used to measure the cytotoxicity, as assessed by growth inhibition potential, of taxol and of 6α-hydroxytaxol. The cell lines were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown and maintained as a suspension culture in a RPMI 1640 preparation containing L-glutamine and 10% (v/v) heat-inactivated fetal calf serum (BRL-GIBCO, Rockville, MD); a penicillin–streptomycin solution (Sigma, St. Louis, MO)

was added to achieve final concentrations of 100 units/mL and 100 μg/mL, respectively. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and were passed every 72–96 h to maintain typical cell densities of 4×10^4 to 5×10^4 cells/mL. Under these conditions the doubling times for MOLT-4 cells and for U-937 cells were 21–22 and 17–19 h, respectively.

Cells were transferred to fresh medium and cultured at a density of 2×10^5 cells/mL 24 h prior to drug exposure. Taxol (Sigma or Developmental Therapeutics Program, NCI) or 6α-hydroxytaxol were diluted in ethanol (USP grade) and added to fresh medium (1 mL final volume). One-milliliter aliquots of these drug solutions were combined with 1 mL of MOLT-4 or U-937 cell suspensions containing 6×10^4 cells/well in 24-well plates (Costar 3424, Cambridge, MA). Final medium concentrations of taxol and 6α-hydroxytaxol ranging from 0.10 nM to 1.0 μM were tested; taxane concentrations in the ethanol stock solutions were verified by HPLC and by UV spectrophotometry. Control experiments were conducted with ethanol, but in the absence of taxanes; ethanol content never exceeded 1% (v/v) in any experiment. Incubations in the presence or absence of taxanes were conducted for 72 h, and the inhibition of cellular growth was assessed by cell counting (Elzone 180 instrument, Particle Data, Inc., Elmhurst, IL). Estimation of IC_{50} values from growth inhibition curves was accomplished using the floating E_{max} routine of the computer program SigmaPlot (Jandel Scientific, San Rafael, CA).

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Supplementary Material Available: Tabular data that describe ^1H and ^{13}C assignments and the results of DQCOSY, TOCSY, HMBC, and HMQC experiments plus 1D proton NMR spectra of taxol and of 6α-hydroxytaxol (3 pages). Ordering information is given on any current masthead page.

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