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Isolation, purification, and biological activity of mono- and dihydroxylated paclitaxel metabolites from human feces

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Abstract Three metabolites of the cytotoxic drug paclitaxel (Taxol) were isolated and purified from the feces of cancer patients receiving the agent as an intravenous infusion. The procedures involved sample homogenization in water followed by liquid-liquid extraction with diethyl ether and high-performance liquid chromatography (HPLC). Approximately 1–3.5 mg of each metabolite was obtained from 100 g of feces. As judged from the chromatographic traces of analytical HPLC with ultraviolet (UV) detection at 227 nm, the purity of each compound was > 97%. On-line photodiode-array detection demonstrated that the UV spectrum of the isolated compounds closely resembles that of the parent drug. Mass spectrometry provided evidence that these metabolites are mono- and dihydroxy-substituted derivatives, namely, 6α-hydroxypaclitaxel, 3'-*p*-hydroxypaclitaxel, and 6α,3'-*p*-dihydroxypaclitaxel. The two 6α-hydroxy-substituted metabolites were shown to have lost their cytotoxicity in in vitro clonogenic assays using the A2780 human ovarian carcinoma and the CC531 rat colon-carcinoma tumor cell lines. In addition, the metabolites showed reduced myelotoxic effects as compared with paclitaxel in an in vitro hemopoietic progenitor toxicity assay. Our procedure for the isolation and purification of paclitaxel metabolites in milligram quantities should be useful for testing the biological activities of these compounds and for the preparation of calibration standards essential for pharmockinetics studies.

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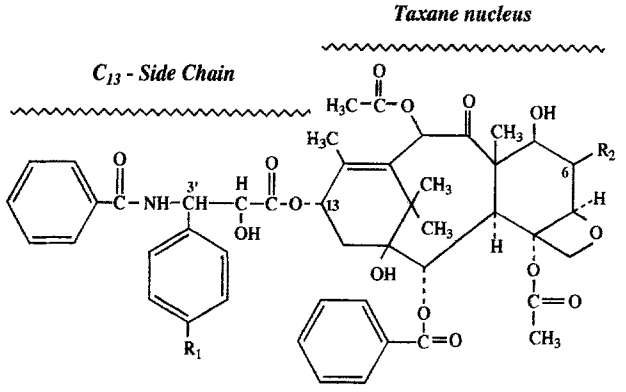
Introduction

Paclitaxel [Taxol; 5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2R,3S)-*N*-benzoyl-3-phenylisoserine] (Fig. 1) is an antineoplastic agent isolated from the bark of the Pacific yew tree, *Taxus brevifolia* [20]. The compound exerts its cytotoxic action through a unique mechanism of action, that is, the promotion of microtubule assembly and stabilization by prevention of depolymerization [5,16,17]. In clinical studies, paclitaxel has shown considerable activity against a variety of human malignancies, including platinum-resistant ovarian cancer [11], breast cancer [4], non-small-cell lung cancer [2], and leukemia [15].

The pharmocokinetics of paclitaxel in humans has been the subject of various studies [1,6,9,10,21]. Although it was initially reported that no metabolites could be detected in plasma samples of cancer patients receiving the drug [14], recent studies provided substantial evidence for the presence of several putative metabolic compounds [6]. The metabolic fate of paclitaxel in rats and humans has been elucidated in part by the analysis of bile and the use of chromatographic methods, mass spectrometry, and nuclear magnetic resonance spectroscopy [7, 8, 12, 13, 19]. In these studies, 6α-hydroxypaclitaxel was identified as a major metabolite in humans. Furthermore, two other identified metabolites, viz., 3'-*p*-hydroxypaclitaxel and 6α,3'-*p*-dihydroxypaclitaxel, and one unknown metabolic product were detected. The importance of these metabolites in the pharmacology and toxicology of paclitaxel is largely unknown. To address this question, both pharmacokinetics studies on the distribution of these metabolites and investigations of their in vitro activity on the growth of normal cells (e.g., colony-forming



compound	R_1	R_2
metabolite I	OH	OH
metabolite II	OH	H
metabolite III	H	OH
paclitaxel	H	H

Fig. 1 Molecular structures of paclitaxel and isolated metabolites

units in the bone marrow) and tumor cell cultures are required. For such studies, however, the availability of the pure compounds is indispensable.

Since human bile is generally not available and the metabolite concentrations in human plasma are relatively low, we evaluated the feces of patients treated with paclitaxel as a potential source of sufficient quantities (milligrams) of these metabolites. Paclitaxel metabolites could be isolated from human feces by liquid-liquid extraction and were further purified by reversed-phase high-performance liquid chromatogrphic (HPLC) procedures. The purified compounds were characterized by HPLC with photodiode-array detection and by fast atom bombardment-mass spectrometry (FAB-MS). Preliminary data gathered on the cytotoxic activity of the isolated metabolites in in vitro clonogenic assays employing human and rat tumor cell lines are presented in this report.

Materials and methods

Chemicals

Unless otherwise specified, all chemicals and reagents were of analytical grade (methanol and acetonitrile were of Lichrosolv quality) and were purchased from E. Merck (Darmstadt, Germany). Paclitaxel reference compound (batch 80617492D) was obtained from the Bristol-Myers Squibb Company (Princeton, N.J., USA). A stock solution of 1 mg/ml was prepared by dissolving 5 mg of paclitaxel in 5 ml of absolute ethanol. A calibration standard of 1000 ng/ml was prepared by dilution of the stock solution in a mixture of acetonitrile: mehanol: water (AMW; 4:1:5, by vol). Water obtained from a Milli-Q Plus system (Millipore, Milford, Mass. USA) was used throughout.

HPLC instrumentation

The HPLC equipment consisted of two Spectroflow SF400 pumps (Kratos, Ramsey, N.J., USA) a Spectroflow SF757 variable-wavelength detector (Kratos) operating at 227 nm or a 1000S photodiode-array detector (Applied Biosystems, Westwood, N.J., USA), a Rheodyne 7010 injector (Cotati, Calif. USA) or a model T660 automatic sampling device (Kontron, Basel, Switzerland), and a model 5140 solvent programmer (Kipp & Zn., Delft, The Netherlands). Peak detection and integration was achieved using a SP4600 DataJet integrator coupled to a WINner/286 data station (Spectra Physics, San Jose, Calif, USA).

Isolation and purification of metabolites

Human feces was obtained from patients receiving 135 or 175 mg/m^2 of Taxol in a 3- or 24-h infusion and was stored at − 30°C until analysis. Homogenization of 1 part of feces in 10 parts of water was accomplished using an Ystral X1020 homogenizer (Dottingen, Germany). Aliquots of 10 ml of the suspension were extracted twice with 20 ml of diethyl ether. The combined diethyl ether fractions were dried by vacuum concentration in a Speed-Vac Plus SC210A system (Savant, Farmingdale, N.Y., USA) at 43°C. Next, the residues were redissolved in acetonitrile and aliquots of 350 μl were loaded onto a glass column (100 × 3 mm) packed with 5-µm Spherisorb CN material (Chrompack, Middelburg, The Netherlands). The injector was located between pump B, delivering a mobile phase of acetonitrile:water (90:10, v/v), and a T-piece where mixing occurred with the mobile phase (water, 100%) coming from pump A. During this loading time, pumps A and B delivered 85% and 15% of the flow rate (0.4 ml/min), respectively. This lineup enabled on-line dilution of acetonitrile in the sample and trapping of the compounds on top of the HPLC column.

Next, the compounds were eluted using a 20-min linear gradient of B ranging from 15% to 95%. Four taxane-related compounds, including the unchanged drug, were separated from the bulk of endogenous compounds and collected in two separate fractions (F), viz., F1 and F2. A total of 20 separate chromatogrphic runs were required and the combined fractions were frozen at − 70°C and lyophilized using a FDC206 freeze-drying chamber (Savant). The residue from F1 was dissolved in acetonitrile:water (30:70, v/v) and the residue from F2, in acetonitrile:water (53:47, v/v). Next, the compounds were separated by semi-preparative HPLC using a stainless-steel (100 × 10 mm) column packed with 5-µm Spherisorb ODS-2 material (Phase Separations, Queensferry, UK). The compounds were eluted isocratically using the AMW mixture as the mobile phase at a flow rate of 3 ml/min. The separated fractions, containing compounds encoded I, II, III, and IV, were lyophilized, weighted, dissolved in 1 ml of methanol, and stored at − 20°C.

The purity of each metabolite was checked using analytical HPLC as previously described in detail [6, 22]. In short, the HPLC system used a stainless-steel (150 × 4.6 mm) column packed with 5-µm APEX-C_8 material (Jones Chromatography, Littleton, Colo., USA). The mobile phase comprised a mixture of acetonitrile:methanol:0.2 *M* ammonium acetate buffer (pH 5.0; 40:10:50, by vol.) and was pumped at a flow rate of 1 ml/min. Aliquots of 100 μl were injected and detection was achieved by photodiode-array detection.

Fast atom bombardment-mass spectrometry

FAB-MS spectra were obtained from the metabolites dissolved in methanol (ca. 1 mg/ml), added to a glycerol matrix, using

a JMS-SX/SX102A tandem mass spectrometer (Jeol, Tokyo, Japan) with a 6-keV xenon atom beam and 10 kV accelerating voltage.

In vitro clonogenic assays

Human ovarian carcinoma A2780 and rat colon carcinoma CC531 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Ltd., Paisley, Scotland) supplemented with 10% fetal calf serum (Sebak, Aidenach, Germany), penicillin, and streptomycin. A2780 and CC531 tumor cells were plated in 6-well, 60-mm tissue-culture dishes (Costar Corporation, Cambridge, Mass, USA) and allowed to attach for 16 h. Paclitaxel or purified metabolite, dissolved in ethanol and diluted further in DMEM, was added to the culture media to a final concentration of 50 n*M*, and the cells were incubated for a period of 4–72 h at 37°C in 5% CO_2/95% air. The final ethanol concentration was 0.1%. Cells grown in the presence of the vehicle used to dissolve and dilute paclitaxel and the metabolites were used as controls. Next, the medium was removed, the dishes were rinsed with phosphate-buffered saline, and fresh culture medium was added. After 7 days, the medium was removed and the cells were fixed with ethanol and stained with crystal violet. The colony formation was judged by light microscopy and scored if a cluster contained more than 50 cells.

Hemopoietic progenitor toxicity assay

Post-ficoll bone marrow cells (5×10^4) of a healthy human donor were seeded for in vitro colony formation (GM-CFU) in 1 ml of semisolid medium (0.8% methylcellulose) in 9.6-cm^2 dishes, with the addition of 250 U of human granulocyte/macrophage colony-stimulating factor/ml (hGM-CSF), 50 ng of human interleukin 3 (IL-3)/ml, and 5% fetal calf serum as described elsewhere [18]. Increasing amounts of paclitaxel or purified metabolite were added to the culture media as described above. After 13 days the number of colonies larger than 50 cells were counted.

Results and discussion

Isolation of metabolites

The HPLC profiles of the diethyl ether extracts from the fecal samples obtained from patients before and after paclitaxel administration are shown in Fig. 2. Five major peaks were observed in the chromatograms. However, one of these peaks (x) was also present in blank fecal specimens. This compound was unstable and its UV spectrum was very different from that of paclitaxel. Hence, it was considered to be an endogenous compound and isolation was not attempted. As determined by on-line photodiode-array detection, compounds encoded I, II, III, and IV showed a UV spectrum very similar to that of paclitaxel reference compound. These were considered putative metabolites and were isolated.

Liquid-liquid extraction with dethyl ether provided a first crude separation from most hydrophilic endogenous compounds. After evaporation of the organic solvent the residue contained substantial amounts of lipophilic, intensively UV/visible (VIS)-absorbing compounds, which were strongly retained on the Spherisorb CN column. By gradient elution, paclitaxel and the putative metabolites eluted prior to the bulk of these interferences and could be collected in two fractions containing either compounds I and II or compound III and IV. Final separation was achieved by semipreparative HPLC and, after drying of the collected fractions by vacuum concentration, the residues were dissolved in 1 ml of methanol.

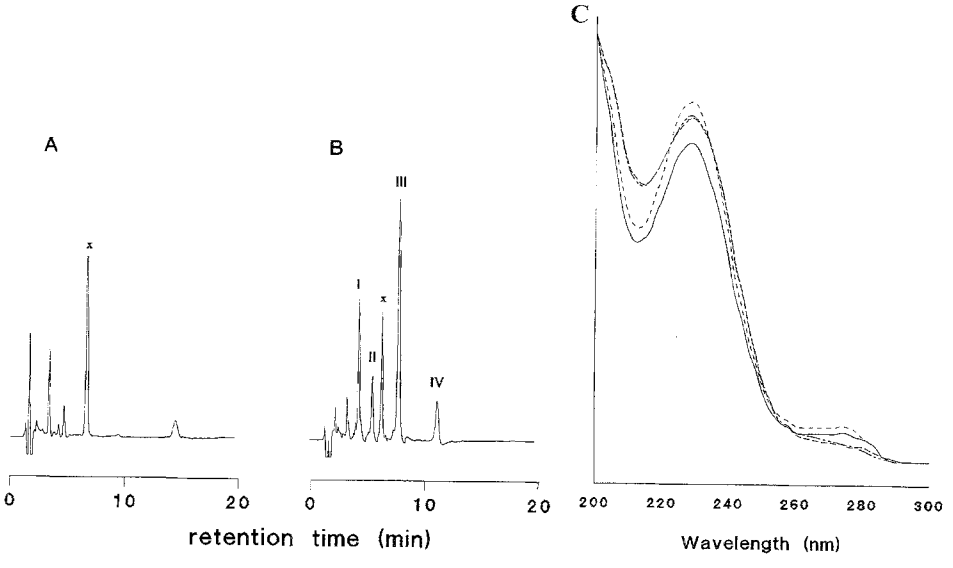


Fig. 2A,B HPLC chromatograms (detection, 227 nm) of diethyl ether extracts of patients fecal homogenates **A** before and **B** after paclitaxel treatment. *Peak IV* represents the parent drug; *Peaks I, II, and III* correspond to metabolites I, II, and III, respectively. The retention times recorded for metabolites I, II, and III and for paclitaxel were 4.0, 5.1, 7.4, and 10.6 min, respectively. *Inset*: UV spectra of paclitaxel and the isolated metabolites. ...., IV (paclitaxel); ——, I (6α,3′-*p*-dihydroxypaclitaxel); - - -, II (3′-*p*-hydroxypaclitaxel); –·–·, III (6α-hydroxypaclitaxel)

Purity of metabolites

Analytical HPLC of the purified metabolites with UV detection at 227 nm revealed the presence of only trace levels of other UV-absorbing compounds. As judged from weight measurements of the residues, the amount of each metabolite obtained was 1.60, 1.05, 3.50, and 1.05 mg of compound I, II, III, and IV, respectively. By calculating the peak areas of each putative metabolite relative to the peak area of a paclitaxel reference solution and assuming similar extinction coefficients at 227 nm, the estimated amount of isolated metabolite was 1.57, 1.02, 3.47, and 1.04 mg for compound I, II, III, and IV, respectively. Therefore, we conclude that non-taxane-related impurities (e.g., salts) accounted for less than 3% of the purified products. These amounts were obtained from a single portion (approx. 100 g) of human feces collected at 15 h after drug administration. The recovery yield of the complete procedure was approx. 50%.

Structural identification of metabolites

Since all major metabolic products in humans have been characterized in previous studies by mass spectrometry and nuclear magnetic resonance spectroscopy [3, 8, 13], identification of the purified products was restricted to the use of HPLC with photodiode-array (PDA) detection and FAB-MS. The elution order of the compounds indicated that product I had the most hydrophilic and product IV the least hydrophilic structure. By PDA detection, the characteristic taxane UV spectrum was observed for all products, exhibiting an absorbance maximum at 227 nm. The UV spectrum of compound III was superimposable with that of paclitaxel, whereas compounds I and II displayed a slight intensification of the secondary absorbance maximum at 275 nm (Fig. 2). FAB-MS data showed protonated molecular ions ($[M + H]^+$) at m/z 886, m/z 870, m/z 870, and m/z 854 for compound I, II, III, and IV, respectively.

On the basis of the FAB-MS data, the UV spectrum, and the identical HPLC retention time in comparison with paclitaxel reference compound, compound IV was identified as the unchanged drug. The increase of 16 and 32 mass units over the protonated molecular ion of paclitaxel (reference compound) indicated the introduction of 1 and 2 oxygen atoms. Metabolite I was identified as 6α,3-*p*-dihydroxypaclitaxel on the basis of the fragments observed at m/z 302 $\{[(C13 \text{ side chain} + 16) + H]^+\}$ and m/z 585 $(\{[\text{taxane nucleus}) + 16] + H\}^+)$, which suggest that hydroxylation has taken place in both the side chain attached to the C13 of the taxane nucleus as well as in the taxane nucleus itself. Prominent fragments of metabolite II included m/z 302 $\{[(C13 \text{ side chain} + 16) + H]^+\}$ and m/z 569 $\{[(\text{taxane nucleus}) + H]^+\}$, thus corresponding to the metabolite 3-*p*-hydroxypaclitaxel. The mass spectrum of metabolite III revealed fragment ion peaks at m/z 286 $\{[(C13 \text{ side chain}) + H]^+\}$ and m/z 585 $(\{[(\text{Taxane nucleus}) + 16] + H\}^+)$ and, hence, was identified as 6α-hydroxypaclitaxel.

In vitro clonogenic assays

When exposed to 50 n*M* of paclitaxel, both the human ovarian carcinoma (A2780) and the rat colon carcinoma (CC531) cell line displayed a time-dependent drug sensitivity, with the human A2780 cells being more sensitive than the CC531 rat tumor cells (Fig. 3).

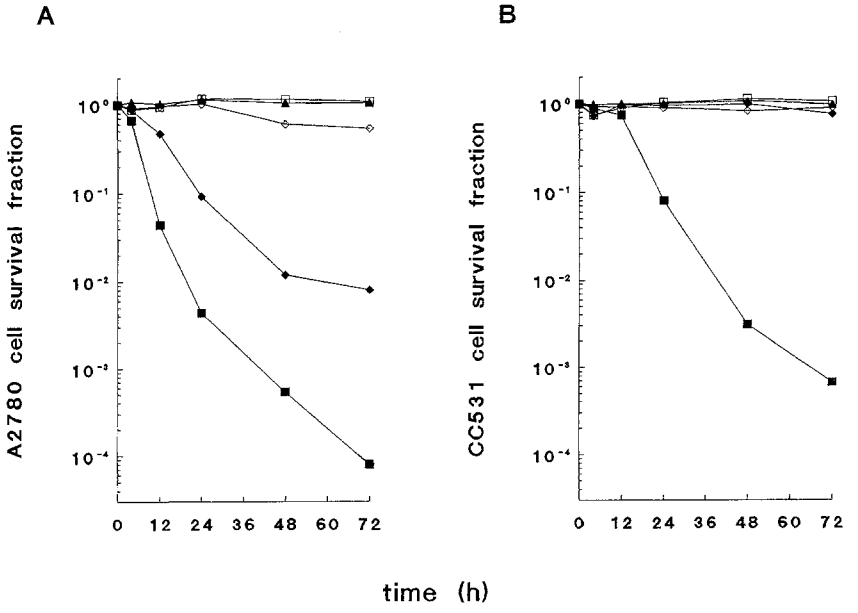


Fig. 3A, B Survival of **A** human A2780 ovarian carcinoma and **B** rat CC531 colon carcinoma cells after exposure to 50 n*M* paclitaxel or purified metabolite for up to 72 h ($n = 3$). ■, IV (paclitaxel); □, I (C6α,C3′-dihydroxypaclitaxel); ◆, II (C3′-hydroxypaclitaxel); ◇, III (C6α-hydroxypaclitaxel); ▲, control

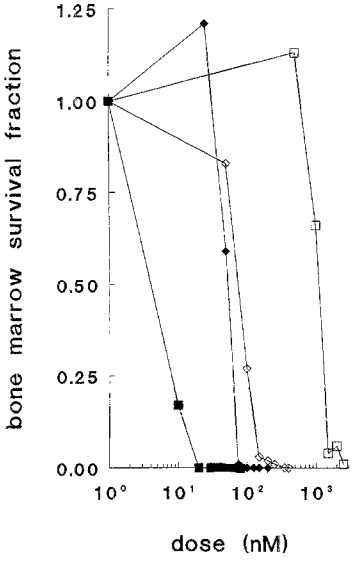


Fig. 4 Survival of normal human bone marrow cells after exposure to different concentrations of paclitaxel or purified metabolite ($n = 2$). ■, IV (paclitaxel); □, I (C6α,C3′-dihydroxypaclitaxel); ◆, II (C3′-hydroxypaclitaxel); ◇, III (C6α-hydroxypaclitaxel); ▲, control

Furthermore, it was observed that no growth inhibition of A2780 or CC531 cells occurred in the presence of 50 n*M* of the 6α-hydroxy-substituted metabolites I and III. Metabolite II displayed differential cytotoxicity against the two cell lines; whereas this compound was inactive against the CC531 tumor cell line, it retained some activity against the A2780 cells.

Hemopoietic progenitor toxicity assay

Similar to the results obtained in the clonogenic assays with tumor cells, the introduction of hydroxy functions in the paclitaxel molecule reduces myelotoxic effects in vitro on bone marrow cells. The mean dose at which the inhibition of colony formation is 50% as compared with untreated cells (IC_{50}) was 4 n*M* for paclitaxel, 60 n*M* for metabolite II, 80 n*M* for metbolite III, and 1000 n*M* for metabolite I (Fig. 4).

In conclusion, we report a useful technique for the isolation and purification of human paclitaxel metabolites in milligram quantities. The amounts of each metabolite that can be isolated in this manner will facilitate their use in future studies on paclitaxel metabolism and pharmocokinetics.

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