Computational Model of Intracellular Pharmacokinetics of Paclitaxel

HYO-JEONG KUH, SEONG HOON JANG, M. GUILLAUME WIENTJES, and JESSIE L.-S. AU

College of Pharmacy, Ohio State University, Columbus, Ohio

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Abstract

The intracellular pharmacokinetics of paclitaxel is closely related to its pharmacodynamics. Although drug transport across the cell membrane and extracellular and intracellular drug binding have been shown to affect intracellular drug accumulation, their quantitative relationship is unknown. This study was designed to establish a mathematical model for computing the intracellular paclitaxel pharmacokinetics. As a starting point, the model assumes drug transport into and out of cells via passive diffusion. Experimental data on the intracellular pharmacokinetics of [3H]paclitaxel were obtained using monolayer cultures of human breast MCF7 tumor cells, which have negligible expression of the mdr1 P-glycoprotein. The results indicate that, in addition to drug binding and microtubule concentration, changes in cell number due to cell growth and drug effects also affected intracellular drug accumulation. A kinetic model was developed to describe several concomitant processes: 1) saturable drug binding to extracellular proteins, 2) saturable and nonsaturable drug binding to intracellular components, 3) time- and concentration-dependent drug depletion from culture medium, 4) cell density-dependent drug accumulation and cell density on the kinetics of drug accumulation and efflux. This model was used to predict the effects of changing several parameters (number and binding affinity of intracellular binding sites, free fraction, and concentration of drug in extracellular fluid) on intracellular drug accumulation. In conclusion, the computational model of intracellular paclitaxel pharmacokinetics provides the means to predict drug concentration in cells.

Paclitaxel, one of the most important anticancer drugs developed in the past two decades, is active against multiple types of human solid tumors (Rowinsky, 1993). Paclitaxel enhances tubulin polymerization, promotes microtubule assembly, binds to microtubules, stabilizes microtubule dynamics, induces mitotic block at the metaphase/anaphase transition, and induces apoptosis (Parness and Horwitz, 1981; Manfredi et al., 1982; Jordan et al., 1993, 1996; Derry et al., 1995). The intracellular concentration of paclitaxel is critical for its pharmacological effect. Drug resistance in several resistant sublines is correlated with reduced intracellular drug accumulation compared with the sensitive parent cell lines (Lopes et al., 1993; Bhalla et al., 1994; Jekunen et al., 1994; Riou et al., 1994; Speicher et al., 1994). One of the challenges regarding the clinical use of paclitaxel is the identification of optimal treatment schedules; multiple treatment schedules with different infusion duration (1, 3, 24, and 96 h) and different treatment frequency (daily, weekly, and every 3 weeks) are under evaluation in patients. The difficulty is in part due to the lack of a precise understanding of the pharmacodynamics of paclitaxel, i.e., drug effect as a function of drug concentration and treatment duration. For example, the importance of treatment duration on the antitumor effect of paclitaxel has been controversial; some studies in cultured cells indicate that prolonging the treatment duration did not enhance the drug effect (Cohen and Duke, 1984; Roberts et al., 1990), whereas other studies indicate the opposite (Rowinsky et al., 1988; Liebmann et al., 1993; Lopes et al., 1993; Milas et al., 1995). We have shown that this controversy is likely due to the delayed cytotoxicity of paclitaxel that is exhibited after termination of treatment. The delayed effect is due in part to the slow manifestation of apoptosis and in part to the slow release of paclitaxel from its intracellular binding sites (Au et al., 1998). These findings indicate that the elucidation of paclitaxel phar-
macrodynamics requires a better understanding of its intracellular pharmacokinetics on a quantitative level. For example, the intracellular drug concentration-time profile is needed to predict the drug effect at various time intervals during and after drug administration.

Several laboratories, including ours, have studied various aspects of intracellular pharmacokinetics of paclitaxel in cultured cells, such as the binding of paclitaxel to extracellular and intracellular macromolecules (Manfredi et al., 1982; Jordan et al., 1993; Song et al., 1996) and the effect of overexpression of the multidrug resistance of P-glycoprotein (Pgp) on drug efflux (Bhalla et al., 1994; Speicher et al., 1994). Although these studies have led to a better understanding of the determinants of intracellular paclitaxel pharmacokinetics on a conceptual level, they do not provide the means to depict quantitatively how changes in these determinants will alter intracellular pharmacokinetics. For example, it is known that: 1) changes in tubulins alter drug binding and accumulation in cells (Haber et al., 1995; Dumontet et al., 1996; Dumontet and Sikic, 1999); 2) the presence of Cremophor micelles decreases the free fraction of paclitaxel available to enter cells (Knemeyer et al., 1998); and 3) displacement of paclitaxel from plasma protein binding sites by other highly protein-bound drugs such as cisplatin increases the free fraction. However, because the quantitative relationship between these determinants and intracellular drug accumulation is unknown, it is not possible to design treatment schedules to accommodate changes in the determinants.

The goal of this study was to establish a computational model of intracellular paclitaxel pharmacokinetics that could be used to quantify the relative importance of various determinants. As a first study, the model was developed for a system where drug efflux does not involve active drug transport by the Pgp efflux pump, and the required experimental data were obtained using human breast adenocarcinoma MCF7 cells, which have negligible Pgp expression (Fairchild et al., 1990; Li et al., 1998).

Materials and Methods

Chemicals and Reagents. Paclitaxel was a gift from Bristol-Myers Squibb Co. (Wallington, CT) and the National Cancer Institute (Bethesda, MD); 3H-paclitaxel (specific activity, 19.3 Ci/mmol) from the National Cancer Institute; and PSC833 from Novartis Inc. (Summit, NJ). 7-Epitaxol was purchased from Hauser Chemicals (Boulder, CO); cefotaxime sodium from Hospoht-Roussel (Somerville, NJ); gentamicin from Solo Pak Laboratories (Franklin Park, IL); all other cell culture supplies including Versene from Life Technologies (Grand Island, NY); Solvable tissue gel solubilizer and Atomlight scintillation fluid from DuPont Biotechnology Systems (Boston, MA); and all other chemicals and β-tubulin monoclonal antibody from Sigma (St. Louis, MO). All chemicals and reagents were used as received. 7-Epitaxol was >99.2% pure, as determined by high pressure liquid chromatographic (HPLC) analysis.

Cell Culture. Human breast MCF7 tumor cells were a gift from Dr. Kenneth Cowan (National Cancer Institute). Cells were maintained in RPMI-1640, supplemented with 9% heat-inactivated fetal bovine serum, 2 mM glutamine, 90 µg/ml gentamicin, and 90 µg/ml cefotaxime sodium, in a humidified atmosphere at 37°C and 5% CO2. For experiments, cells were harvested from confluent cultures using trypsin and resuspended in fresh medium before plating. Cells with greater than 90% viability, as determined by trypsin blue exclusion, were used. The doubling time of MCF7 cells, during the exponential growth phase, was 24 h.

Cell Volume Measurement. The volume of MCF7 cells in the exponential growth phase was determined using the Samba Image Analyzer 4000 (Imaging Products International, Inc., Chantilly, VA). Because the drug-induced reorganization of microtubules has no effect on cell volume (Brown et al., 1985; Mills, 1987), cell volume was determined using untreated cells. Cells were harvested with trypsin, stained with toluidine blue (0.2% in 10% methanol), and diluted with PBS. The Samba 4000 quantified the distance as the number of pixels, i.e., 164 pixels per 200 μm microfield. The maximum (L) and minimum (W) diameters of a cell, which were determined by counting the number of pixels and converting the value to micrometers, were used to calculate the cell volume. Equation 1 describes the calculation of the volume (V) of ellipsoid cells. MCF7 cells in the log growth phase showed an average volume of 2.00 ± 0.431 μm3/106 cells (mean ± S.D.; n = 232; median, 2.11 μl).

\[
V = \frac{4}{3} \pi L \cdot W^2
\]

Uptake and Efflux of Paclitaxel. Cells were plated at densities of 10^5 to 10^6 cells/well in 1 ml of culture medium in six-well plates. One day after seeding, the medium was replaced with 1 ml of medium containing [3H]paclitaxel. Nonradioabeled paclitaxel was added when necessary. The final specific activity of [3H]paclitaxel ranged from 0.04 to 19.3 Ci/mmol. Drug uptake experiments used drug concentrations (total concentration) that are within the therapeutic range of 1 to 5000 nM (Kearns et al., 1995). To determine drug efflux, cells were treated with 10 nM [3H]paclitaxel for 24 h, then washed twice with 1 ml of ice-cold drug-free medium, followed by incubation in drug-free medium with agitation every 15 min for 2 h and every hour thereafter. For both uptake and efflux studies, aliquots (100 µl) of medium were removed at predetermined times. After the remaining medium was aspirated, cells were washed twice with 0.25 ml of ice-cold Versene and then harvested as a suspension after trypsinization. Cell number was measured by hemocytometer or by Coulter Counter (Coulter Electronics Inc., Hialeah, FL) after a 20-fold dilution with Isotone (Coulter Electronics Inc.). Samples were dissolved in 0.5 ml of Solvable tissue gel solubilizer, mixed with 10 ml of Atomlight, and processed by liquid scintillation counting.

A separate study in our laboratory using differential centrifugation shows that <5% of the cell-associated paclitaxel was accounted for by the paclitaxel located in cell membrane and mitochondria fractions (J. Kim and J. L.-S. Au, unpublished observations). These data indicate a negligible amount of membrane-associated paclitaxel. Hence, the total cell-associated drug concentration was taken as equal to the intracellular concentration.

Sample Extraction and Analysis. Paclitaxel and its reversible epimerization product, 7-epitaxol, were extracted using ethyl acetate and ammonium acetate buffer (0.01 M, pH 5.0). The latter was added to minimize epimerization (Leslie et al., 1993). The organic extract was evaporated to dryness. After being reconstituted with 16% acetonitrile in ammonium acetate, the sample was loaded on BondElut CN solid-phase extraction cartridges (Varian, Harbor City, CA) pre-equilibrated sequentially with 2 ml of acetonitrile, 2 ml of methanol, and 3 ml of ammonium acetate buffer. After being washed with 2 ml of ammonium acetate buffer and 2 ml of 25% acetonitrile in the same buffer, the analytes were eluted with 70% acetonitrile in water. The eluent was evaporated to dryness and reconstituted in the HPLC mobile phase (49% acetonitrile in water). The extraction recovery was >90% for both culture medium and cell samples. An aliquot was analyzed by HPLC using a reversed phase µBondpak C18 column (Waters Association, Milford, MA) and UV absorbance at 229 nm. The flow rate of the mobile phase was 1 ml/min, and the retention times for paclitaxel and 7-epitaxol were 13.6 and 21.9 min, respectively. More than 95% of the radioactive material recovered in the HPLC-eluting fractions corresponded to paclitaxel and 7-epitaxol. 7-Epitaxol accounted for less than 10% of the total radioactivity at 4 h. Because paclitaxel and 7-epitaxol together accounted for almost all of the recovered radioactivity and because paclitaxel and 7-epitaxol are pharmacologically equivalent, with identical...
microtubules binding affinity and cytotoxicity (Ringel and Horwitz, 1987), the total recovered radioactivity was considered equivalent to paclitaxel without further correction in subsequent studies.

Confirmation of Negligible Pgp-Mediated Paclitaxel Efflux in MCF7 Cells. The MCF7 cells used in this study are known to have negligible Pgp expression (Fairchild et al., 1990). A separate study using Western blot analysis confirmed that the Pgp level in these cells was barely detectable (Li et al., 1998). To determine whether Pgp-mediated efflux significantly contributed to the efflux of paclitaxel, we compared the intracellular concentration-time profiles in the absence or presence of a known Pgp inhibitor, PSC833 (Boesch et al., 1991). A preliminary study showed that PSC833, at 0.5, 1, and 5 μg/ml, did not affect the growth of MCF7 cells for at least 5 days. Subsequent studies used 1 μg/ml PSC833. In the uptake study, PSC833 was administered with paclitaxel. In the efflux study, cells were first treated with paclitaxel for 24 h; the paclitaxel-loaded cells were then placed in PSC833-containing medium.

Analysis of Total and Polymerized Tubulin. Total (free plus polymerized) and polymerized tubulin were analyzed as previously described (Thrower et al., 1991), with the exception that cells were lysed by four to five cycles of freezing, thawing, and vortexing. More than 90% of the cells were lysed, as indicated by the uptake of trypan blue dye. Tubulin was analyzed by an enzyme-linked immunosorbent assay using a monoclonal antibody to β-tubulin (IgG, Tub2.1). Bovine brain tubulin was used as the tubulin standard due to the unavailability of human tubulin. Hence, we were not able to determine the absolute concentration/amount of tubulin in human cells. Accordingly, changes in the tubulin concentration/amount were reported as changes relative to the control value.

Model Development. We constructed an intracellular pharmacokinetic model to describe the factors that determine the kinetics of paclitaxel uptake, binding, and efflux from cells. The model included: 1) saturable binding of paclitaxel to proteins in the extracellular compartment (Song et al., 1996), 2) saturable and nonsaturable binding of paclitaxel to cellular components (Manfredi et al., 1982), 3) time- and concentration-dependent changes in microtubule mass (Jordan et al., 1993; Derry et al., 1995), and 4) time- and concentration-dependent changes in cell number (see Results). We assumed that a) drug uptake is by passive diffusion because sodium azide treatment, which depletes ATP, has minimal effect on intracellular drug accumulation (Manfredi et al., 1982); b) drug efflux is by passive diffusion because Pgp-mediated drug efflux was insignificant in MCF7 cells (see Results); and c) only free drug participates in the uptake and efflux processes.

Equations 2 and 3 are the differential mass balance equations that describe changes in the amounts of paclitaxel in cells (Amttotal,c) and in medium (Amttotal,m), as a function of time. Ctotal,c and Ctotal,m are the total (i.e., free plus bound) drug concentrations in cells and medium, respectively; Cfree,c and Cfree,m are the free drug concentrations in cells and medium, respectively; V, is the cell volume; as shown below, it changed with time due to cell proliferation or drug effect. Vm is the volume of medium; it remained constant (i.e., <10% after 24 h). CLf is the clearance of free drug by passive diffusion, on a per cell basis. Note that CLf did not differ with initial extracellular drug concentration (see Table 3), indicating that the paclitaxel effect, e.g., increases in microtubules, does not affect the diffusional permeability.

\[ \frac{dA_{\text{total},c}}{dt} = V_c \cdot \frac{dC_{\text{total},c}}{dt} + C_{\text{total},c} \cdot \frac{dV_c}{dt} = -CL_c \cdot \text{cell number} \cdot C_{\text{free},c} + CL_c \cdot \text{cell number} \cdot C_{\text{free},m} \]  

\[ \frac{dA_{\text{total},m}}{dt} = V_m \cdot \frac{dC_{\text{total},m}}{dt} = -CL_m \cdot \text{cell number} \cdot C_{\text{free},m} + CL_m \cdot \text{cell number} \cdot C_{\text{free},c} \]  

Cfree,c and Cfree,m are related to Ctotal,c and Ctotal,m, respectively, as depicted in eqs. 4 and 5. Note that the volume fractions for free drug are the same as for total drug.

\[ C_{\text{total},c} = C_{\text{free},c} + \frac{B_{\text{max},c} \cdot C_{\text{free},c}}{K_d,c + C_{\text{free},c}} + \text{NSB} \cdot C_{\text{free},c} \]  

\[ C_{\text{total},m} = C_{\text{free},m} + \frac{B_{\text{max},m} \cdot C_{\text{free},m}}{K_d,m + C_{\text{free},m}} \]  

where Bmax,c and Kd,c are the Michaelis-Menten constants of drug binding to cellular components, and Bmax,m and Kd,m are the constants for drug binding to proteins in medium. NSB is the proportionality constant for nonsaturable binding sites in cells. A previous study showed that there is no nonsaturable binding of paclitaxel in cell culture medium (Song et al., 1996).

Changes in cell number were represented by changes in \( V_c \). \( V_c \) increased with time at low initial total extracellular drug concentrations (1 and 10 nM) due to continued cell proliferation and decreased with time at high initial total extracellular drug concentrations (100 and 1000 nM) due to the antiproliferative and/or cytotoxic drug effects. Equation 6 describes the time-dependent changes in total cell volume at different extracellular drug concentrations. \( V_{\text{one cell}} \) is the average cell volume, ICN is the initial cell number at time 0, and \( k_{\text{cell number}} \) is the rate constant for changes in cell number. Note that the values of \( k_{\text{cell number}} \) depend on the pharmacological effect of paclitaxel. At low paclitaxel concentrations, cells continue to proliferate, resulting in positive values for \( k_{\text{cell number}} \). At higher drug concentrations (i.e., 100 and 1000 nM), cell number decreases, resulting in negative values for \( k_{\text{cell number}} \).

\[ V_c = V_{\text{one cell}} \cdot \text{cell number} = V_{\text{one cell}} \cdot ICN \cdot e^{\left(\frac{A_{\text{total},c}}{V_c} \cdot t\right)} \]  

Microtubule mass is enhanced by paclitaxel in a concentration-dependent manner (Jordan et al., 1993). We found that treatment with 1000 nM paclitaxel resulted in a linear enhancement with time in tubulin concentration up to the last time point at 24 h. This relationship is described by eq. 7. \( B_{\text{max},c} \) and \( B_{\text{max},c,\text{initial}} \) are the maximal saturable binding sites at time t and 0, respectively, and \( k_{\text{Bmax},c} \) is the rate constant for increases of \( B_{\text{max},c} \). Note that \( k_{\text{Bmax},c} \) varied with drug concentration (see Results).

\[ B_{\text{max},c}(t) = B_{\text{max},c,\text{initial}} \cdot \left(1 + k_{\text{Bmax},c} \cdot t\right) \]  

Substitution and rearrangement of eqs. 2 through 7 yielded eqs. 8 and 9, which describe the time-dependent changes in intracellular and extracellular drug concentrations, respectively, as a function of cell volume, binding affinity, and binding capacity.
where \( A = K_{d,m} + B_{\text{max,m}} - C_{\text{total,m}} \) and \( B = (1 + \text{NSB}) \cdot K_{d,c} + B_{\text{max,c}} - C_{\text{total,c}} \).

Equations 8 and 9 were used with the numerical integration method of WINNONLIN (SCI Software, Lexington, KY) to simulate the intracellular and extracellular drug concentration-time profiles.

**Determination of Model Parameters.** Several model parameters were determined experimentally, as follows: \( B_{\text{max,m}} \) and \( K_{d,m} \) were determined by analyzing our previously published data on paclitaxel binding to proteins in cell culture medium (Song et al., 1996) using eq. 5; \( h_{\text{cell number}} \) was calculated for each initial \( C_{\text{total,m}} \) as the slope of the log-linear plot of [cell number] versus [time]; and \( k_{\text{B}} \) was calculated for each initial \( C_{\text{total,c}} \) as the slope of the plot of [concentration of total tubulin] versus [time].

The remaining parameters (i.e., \( B_{\text{max,c}}, K_{d,c}, \text{NSB}, \) and \( CL_f \)) were obtained by model simulation. For these parameters, we first obtained initial estimates by analyzing the data at 4 h, which was the time point when the intracellular and extracellular drug concentrations approached equilibrium with <10% changes within 2 h. We assumed that at this time, \( C_{\text{free,c}} = C_{\text{free,m}} \), which in turn was calculated from the experimentally determined \( C_{\text{total,m}} \) using eq. 5. Analysis of the plot of \( C_{\text{total,c}} \) versus \( C_{\text{free,c}} \) at 4 h, using eq. 4, provided the initial estimates of \( B_{\text{max,c}}, K_{d,c}, \) and NSB. Simultaneously fitting eqs. 8 and 9 to the experimentally determined \( C_{\text{total,c}} \) and \( C_{\text{total,m}} \)-time profiles using these initial estimates provided the initial estimates of \( CL_f \). The initial estimates were substituted into eqs. 8 and 9 to generate model prediction. The values of the parameters were altered until the model-predicted data closely aligned with the experimental data. The values that yielded the best fits between simulated data and experimental data, as indicated by the lowest sum of squared errors, were identified as the final model parameters.

**Validation of the Kinetic Model.** The intracellular pharmacokinetic model was used to predict the effect of cell density on drug accumulation and the effect of the intracellular-to-extracellular concentration gradient on drug efflux. The model-predicted data were then compared with experimental results to evaluate the validity of the model.

**Application of Intracellular Pharmacokinetic Model.** The intracellular pharmacokinetic model was used to demonstrate the effects of changing several parameters (i.e., number and dissociation constant of intracellular binding sites, free fraction of drug in extracellular fluid, and extracellular drug concentrations) on intracellular drug concentrations. These simulations were accomplished by altering the values of \( B_{\text{max,c}}, K_{d,c}, B_{\text{max,m}} \)-to-\( K_{d,c} \) ratio, and initial \( C_{\text{total,m}} \), respectively. Simulations were conducted for initial cell density of \( 10^6 \) cells/ml of medium volume.

**Computer Simulation.** For model fitting and simulations, we used WINNONLIN with 1/concentration as the weight.

**Results**

**Verification of Model Assumption.** Paclitaxel is a substrate for Pgp but not for multidrug resistance-associated protein, another drug efflux membrane protein (Breuninger et al., 1995). The assumption of a negligible Pgp-mediated drug transport in MCF7 cells was verified with an inhibitor study. Treatment of MCF7 cells with the Pgp inhibitor PSC833 at 1 \( \mu \)g/ml did not alter the intracellular accumulation of paclitaxel. For the control and the PSC833-treated cells, the areas under the intracellular concentration-time profiles were 79 ± 8 and 84 ± 7 \( \mu \)M·h, respectively (mean ± S.D., \( n = 3 \)). A separate study showed that PSC833 at this concentration completely inhibited the Pgp-mediated efflux in the \( mdr1 \)-transfected subline of the MCF7 cells (i.e., BC19 cells), which showed a 9-fold higher Pgp expression and 60% lower drug accumulation (Jang et al., 1998; Li et al., 1998). These data rule out significant Pgp-mediated efflux and sup-

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**Fig. 1.** Intracellular and extracellular concentrations of paclitaxel during uptake. MCF7 cells were incubated with 1 to 1000 nM paclitaxel. The concentration of paclitaxel in cells (left panel) and culture medium (right panel) were monitored for 24 h. Note the different units for intracellular and extracellular concentrations. Symbols are the experimental data. Lines are computer-simulated data using eqs. 8 and 9. Mean ± S.D. (\( n = 3 \)). All S.D. values are smaller than the symbols.
port the assumption that paclitaxel is removed from MCF7 cells by passive diffusion.

Kinetics of Paclitaxel Accumulation and Binding. Figure 1 shows the kinetics of paclitaxel accumulation in MCF7 cells. The extensive drug accumulation in cells, indicated by the high intracellular-to-extracellular concentration ratios (Table 1), resulted in significant depletion of paclitaxel from the medium, i.e., >70% depletion at 1 and 10 nM and ~40% depletion at 1000 nM. The depletion occurred even though the volume of the drug-containing medium was more than 500 times the cell volume, a condition that is commonly used in cell culture studies. The intracellular concentration increased with time and approached plateau levels between 1 and 4 h, with the longest time to reach plateau levels at the lowest extracellular concentration. The intracellular-to-extracellular concentration ratio at 4 h decreased 13-fold when the extracellular concentration increased from 1 to 1000 nM. However, when the extracellular concentration was further elevated to 2000 and 5000 nM, the intracellular-to-extracellular concentration ratio remained relatively constant at ~75. As shown below, these concentration-dependent changes in concentration ratios are due to the saturation of the saturable binding sites at higher extracellular drug concentrations and to the linear increase of nonsaturable binding with extracellular concentration.

Table 2 shows the relationship between free and bound concentrations of paclitaxel. At 1 to 1000 nM extracellular concentrations, more than 99.8% of the intracellular concentration was represented by the drug bound to serum proteins in the medium. Our model-predicted and the experimentally verified; the model-predicted and the experimentally determined intracellular and extracellular concentration ratios (Table 2), resulted in significant depletion of paclitaxel from the medium, ranging from a 21% depletion for a cell density of 0.08 \times 10^6 cells/ml to a 90% depletion at a cell density of 2 \times 10^6 cells/ml when cells were treated with 10 nM initial extracellular concentration. The depletion of drug in the medium would in turn result in a lower drug accumulation in cells plated at a high density. This prediction was experimentally verified; the model-predicted and the experimentally determined intracellular concentration, obtained at several cell densities ranging from 0.13 to 1.3 \times 10^6 cells/ml, deviated by 7.5 ± 3.2% (range, 2–11%; Fig. 2). A practical solution for reducing drug depletion while maintaining a desired plateau density would be to increase the volume of culture medium. Our model predicted that increasing the medium volume by 10-fold would reduce the extent of drug depletion in

### TABLE 1

<table>
<thead>
<tr>
<th>Initial C\text{total,m}</th>
<th>Initial Cell Density</th>
<th>Cell Density at 24 h</th>
<th>C\text{total,m} at 4 h</th>
<th>C\text{total,c} at 4 h</th>
<th>C\text{total,m} \text{Ratio at 4 h}</th>
<th>C\text{total,m} at 24 h</th>
<th>C\text{total,c} at 24 h</th>
<th>C\text{total,m} \text{Ratio at 24 h}</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>(10^6) cells/ml</td>
<td>(nM)</td>
<td>(\mu M)</td>
<td>(nM)</td>
<td>(\mu M)</td>
<td>(nM)</td>
<td>(\mu M)</td>
<td>(nM)</td>
</tr>
<tr>
<td>0 (control)</td>
<td>0.70 ± 0.03</td>
<td>1.36 ± 0.03</td>
<td>N.A.</td>
<td>N.A.</td>
<td>1283 ± 58</td>
<td>0.188 ± 0.09</td>
<td>0.24 ± 0.03</td>
<td>1280 ± 41.5</td>
</tr>
<tr>
<td>1</td>
<td>0.86 ± 0.05</td>
<td>1.17 ± 0.02</td>
<td>0.239 ± 0.012</td>
<td>0.31 ± 0.02</td>
<td>1283 ± 58</td>
<td>0.188 ± 0.09</td>
<td>0.24 ± 0.03</td>
<td>1280 ± 41.5</td>
</tr>
<tr>
<td>10</td>
<td>0.51 ± 0.06</td>
<td>0.63 ± 0.04</td>
<td>4.01 ± 0.23</td>
<td>5.17 ± 0.22</td>
<td>1312 ± 25</td>
<td>2.95 ± 0.32</td>
<td>4.64 ± 0.41</td>
<td>1520 ± 130</td>
</tr>
<tr>
<td>100</td>
<td>0.65 ± 0.04</td>
<td>0.60 ± 0.05</td>
<td>36.2 ± 3.5</td>
<td>33.3 ± 0.33</td>
<td>922 ± 44</td>
<td>30.8 ± 3.2</td>
<td>39.0 ± 2.3</td>
<td>1200 ± 122</td>
</tr>
<tr>
<td>1000</td>
<td>1.04 ± 0.06</td>
<td>0.92 ± 0.04</td>
<td>684 ± 7.66</td>
<td>72.4 ± 0.64</td>
<td>106 ± 2.2</td>
<td>622 ± 28</td>
<td>115 ± 4.4</td>
<td>185 ± 15.1</td>
</tr>
<tr>
<td>2000</td>
<td>0.95 ± 0.08</td>
<td>N.D.</td>
<td>1690 ± 54.2</td>
<td>123 ± 12</td>
<td>72.0 ± 5.3</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5000</td>
<td>1.36 ± 0.12</td>
<td>N.D.</td>
<td>4180 ± 95.3</td>
<td>315 ± 30</td>
<td>75.8 ± 8.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.A., not applicable; N.D., not determined.
TABLE 2
Intracellular and extracellular binding of paclitaxel
The free and bound concentrations of paclitaxel in culture medium (i.e., \(C_{\text{free,m}}\) and \(C_{\text{bound,m}}\)) and in cells (i.e., \(C_{\text{free,c}}\) and \(C_{\text{bound,c}}\)) at 4 h, when \(C_{\text{free,m}}\) was assumed to equal \(C_{\text{free,c}}\), were calculated using eqs. 4 and 5 and model parameters shown in Table 3, for a cell density of 1 \(\times 10^6\) cells/ml.

<table>
<thead>
<tr>
<th>Initial (C_{\text{total,m}})</th>
<th>(C_{\text{free,m}})</th>
<th>(C_{\text{bound,m}})</th>
<th>% Bound in Medium</th>
<th>(C_{\text{bound,c}})</th>
<th>% Bound in Cells</th>
<th>C_{\text{bound,c}}/C_{\text{bound,m}} Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.031</td>
<td>0.158</td>
<td>83.6</td>
<td>378</td>
<td>99.9</td>
<td>2,390</td>
</tr>
<tr>
<td>10</td>
<td>0.328</td>
<td>1.66</td>
<td>83.5</td>
<td>3,820</td>
<td>99.9</td>
<td>2,300</td>
</tr>
<tr>
<td>100</td>
<td>5.37</td>
<td>26.9</td>
<td>83.4</td>
<td>33,000</td>
<td>99.9</td>
<td>1,230</td>
</tr>
<tr>
<td>1,000</td>
<td>159</td>
<td>667</td>
<td>90.7</td>
<td>88,900</td>
<td>99.8</td>
<td>132</td>
</tr>
</tbody>
</table>

TABLE 3
Parameters for the intracellular kinetic model of paclitaxel uptake, binding and efflux
The model parameters were obtained as described in Materials and Methods. For \(B_{\text{max,m}}\), \(K_a\), and \(K_D\), the results represent the mean \(\pm\) S.D. of the values determined from analyzing the four profiles obtained for four initial extracellular drug concentrations (i.e., 1, 10, 100, and 1,000 nM). For \(k_{\text{cell number}}\) and \(k_{\text{Bmax,c}}\), the values were obtained for the four initial extracellular drug concentrations as described in Materials and Methods. For \(k_{\text{Bmax,c}}\), the results represent the mean \(\pm\) S.D. of three experiments at each initial extracellular drug concentration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Extracellular Concentration</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B_{\text{max,m}})</td>
<td></td>
<td>N.A.</td>
</tr>
<tr>
<td>(K_a)</td>
<td></td>
<td>3.94 (\pm) 0.16 nM</td>
</tr>
<tr>
<td>(B_{\text{max,c}})</td>
<td></td>
<td>N.A.</td>
</tr>
<tr>
<td>(K_f)</td>
<td></td>
<td>781 (\pm) 12 nM</td>
</tr>
<tr>
<td>(K_D)</td>
<td></td>
<td>N.A.</td>
</tr>
<tr>
<td>(C_{\text{free,m}})</td>
<td></td>
<td>59.2 (\pm) 3.0 (\mu)M</td>
</tr>
<tr>
<td>(k_{\text{cell number}})</td>
<td></td>
<td>4.93 (\pm) 0.75 h(^{-1})</td>
</tr>
<tr>
<td>1</td>
<td>0.0088 h(^{-1})</td>
<td>N.A.</td>
</tr>
<tr>
<td>10</td>
<td>0.0046 h(^{-1})</td>
<td>N.A.</td>
</tr>
<tr>
<td>100</td>
<td>0.0053 h(^{-1})</td>
<td>N.A.</td>
</tr>
<tr>
<td>1000</td>
<td>-0.0053 h(^{-1})</td>
<td>N.A.</td>
</tr>
<tr>
<td>(k_{\text{Bmax,c}})</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2.47 (\pm) 0.152 (\times) 10(^{-3}) h(^{-1})</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.17 (\pm) 0.208 (\times) 10(^{-3}) h(^{-1})</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>10.8 (\pm) 2.08 (\times) 10(^{-3}) h(^{-1})</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>31.3 (\pm) 4.25 (\times) 10(^{-3}) h(^{-1})</td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable.

TABLE 4
Changes in total and polymerized tubulin
MCF7 cells were treated with paclitaxel. Changes in total and polymerized tubulins (i.e., microtubule), as a function of time and initial extracellular drug concentration \(C_{\text{total,m}}\), are shown. Mean \(\pm\) S.D. of three experiments.

<table>
<thead>
<tr>
<th>(nM)</th>
<th>Time (h)</th>
<th>Total Tubulin % Control</th>
<th>% Microtubule in Untreated Control</th>
<th>% Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>24</td>
<td>100 \pm 26</td>
<td>75.4 (\pm) 6.3</td>
<td>75.4 (\pm) 6.3</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>106 \pm 8</td>
<td>75.9 (\pm) 3.9</td>
<td>74.5 (\pm) 7.8</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>112 \pm 3</td>
<td>83.8 (\pm) 4.2</td>
<td>74.6 (\pm) 3.8</td>
</tr>
<tr>
<td>100</td>
<td>24</td>
<td>126 \pm 10</td>
<td>119 (\pm) 15*</td>
<td>94.7 (\pm) 5.5*</td>
</tr>
<tr>
<td>1000</td>
<td>24</td>
<td>111 \pm 21</td>
<td>106 (\pm) 10*</td>
<td>97.2 (\pm) 9.7*</td>
</tr>
<tr>
<td>1000</td>
<td>24</td>
<td>129 \pm 26</td>
<td>122 (\pm) 19*</td>
<td>95.0 (\pm) 5.2*</td>
</tr>
<tr>
<td>1000</td>
<td>24</td>
<td>175 (\pm) 22*</td>
<td>165 (\pm) 22*</td>
<td>94.1 (\pm) 0.5*</td>
</tr>
</tbody>
</table>

* \(P < .05\) compared with control (unpaired t test).

extracellular concentration of 10 nM. The model-predicted data and the experimental data showed good agreement, with <7% differences (Fig. 2). As would be expected, the efflux half-life was not dependent on the volume of the wash-out medium and remained at about 2.5 h, whereas the fraction of intracellular paclitaxel retained after 24 h was dependent on the volume of the wash-out medium, ranging from 74% in 1 ml to 52% in 4 ml of medium.

To further verify the model and determine the quantitative importance of cell proliferation and tubulin production/polymerization for the intracellular pharmacokinetics of paclitaxel, we compared the experimental data with the model-predicted data obtained by either including or excluding these two processes (Fig. 3). The comparison shows that inclusion of the increase in cell number with time improved the prediction of intracellular concentration at the 1 nM extracellular concentration by 6% at 4 h and 35% at 24 h. The inclusion of the time-dependent increase in tubulin concentration improved the prediction of intracellular concentration at the 1000 nM extracellular concentration by 11% at 4 h and 65% at 24 h. We also evaluated the consequences of neglecting the 10% reduction of the culture medium volume at 24 h due to evaporation; the differences were insignificant (1 and 2.5% difference at 4 and 24 h, respectively; data not shown). In summary, the good agreement between the model-predicted and the experimental data, under various conditions, indicates the validity of the model.

Examples of Application of a Computational Intracellular Paclitaxel Pharmacokinetic Model. The computational intracellular pharmacokinetic model can be used to depict changes in intracellular drug concentration as a function of extracellular concentration, time, number and binding affinity of binding sites, and cell density. To demonstrate its use, we performed the following simulations.

Drug resistance is related to altered expression of tubulins (Haber et al., 1995; Ranganathan et al., 1998), including changes in the amount of total tubulins (∼2-fold increase or decrease in several paclitaxel-resistant sublines of the human uterine MES-SA tumor cells and ∼2-fold increase in a paclitaxel-resistant subline of the murine macrophage-like J774.2 cells; Haber et al., 1995; Dumontet et al., 1996), and possible drug-binding affinity (Dumontet and Sikic, 1999). These biological changes can be expressed mathematically by altering the number \(B_{\text{max,c}}\) and the dissociation constant \(K_{\text{D,c}}\) of the saturatable intracellular binding sites. Figure 4 shows the simulated intracellular concentration-time profiles obtained under these conditions. A 2-fold increase and decrease in \(B_{\text{max,c}}\) resulted in a 66% higher and a 33% lower accumulation at 1000 nM extracellular concentration, respectively, but only a minor effect at 1 nM, i.e., 10% higher and 14% lower accumulation, respectively. A 4-fold increase

the medium, ranging from 2.5% depletion at a cell density of 0.08 \(\times\) 10\(^6\) cells/10 ml to 41% depletion at 2 \(\times\) 10\(^6\) cells/10 ml. This, again, was experimentally confirmed (Fig. 2).

The model was also validated by comparing the kinetics of paclitaxel efflux at different intracellular-to-extracellular concentration gradients. This was accomplished by using different volumes of wash-out medium (i.e., 1, 2, and 4 ml) at a cell density of 1.5 \(\pm\) 0.2 \(\times\) 10\(^6\) cells/well and an initial
in $K_{\text{d,e}}$, resulted in a 32% decrease in drug accumulation at 1 nM extracellular concentration but had a negligible effect at 1000 nM (<5% decrease). These data indicate that the number of binding sites is an important determinant of drug accumulation at high extracellular concentration, whereas the binding affinity is an important determinant at low extracellular concentration.

The free concentration of paclitaxel in extracellular fluid determines the drug entry into cells. The free fraction of paclitaxel in plasma may increase in the presence of other highly protein-bound drugs such as cisplatin, which may displace paclitaxel from plasma protein-binding sites, and may decrease in the presence of Cremophor micelles (Knemeyer et al., 1998). Both scenarios are clinically plausible because combination therapy with cisplatin and paclitaxel is used and because the Cremophor concentrations in plasma attained after an i.v. infusion of the commercially available paclitaxel formulation (i.e., Taxol) are sufficient to form micelles. To quantify the effect of altering the free fraction of paclitaxel in extracellular fluid on intracellular drug accumulation, simulations were performed by altering the extracellular free-to-bound concentration ratio. The results show that a 4-fold decrease in free fraction from 20 to 5% resulted in 37 and 25% reductions in intracellular drug accumulation at 1 and 1000 nM extracellular concentrations, respectively (Fig. 4). These relatively minor changes in intracellular drug accumulation are due to the higher binding affinity of the intracellular binding sites, compared with extracellular binding sites.

Plasma concentrations of paclitaxel attained after an i.v. infusion of a therapeutic dose span a 10,000-fold range, from 1 to 10,000 nM. Figure 5 shows the corresponding intracellular concentrations, spanning a 2000-fold range, from 0.4 to 800 μM. The relationship between extracellular and intracellular paclitaxel concentrations is depicted in Fig. 5.
cellular concentrations consisted of a mixture of linear and nonlinear relationship. At an initial extracellular concentration of ≤100 nM, or before saturation of the saturable intracellular binding that constitutes the major mode of drug binding at this concentration range, intracellular drug concentration increased linearly with extracellular concentration. At extracellular concentrations between 100 and 1000 nM, when the saturable intracellular binding approaches saturation, intracellular drug concentration increased nonlinearly with extracellular concentration. Finally, at the higher concentrations above 1000 nM, when the nonsaturable binding becomes the major mode of intracellular drug binding, intracellular drug concentration increased linearly with extracellular concentration.

Discussion

Relationship Between Cell Density, Tubulin Polymerization/Production, and Drug Accumulation. The intracellular concentration-time profiles, depicted in Fig. 1, showed two unusual features. First, the intracellular paclitaxel concentration attained at 1 and 10 nM extracellular concentrations reached a maximum value at 4 h and subsequently declined by 23 and 10%, respectively, by 24 h (Table 1). Second, the intracellular concentrations attained at 100 and 1000 nM extracellular concentrations continued to increase with time by about 20 and 50% between 4 and 24 h, respectively (Table 1). These profiles differ from the more commonplace situation where intracellular drug concentration increases with time to reach and remain at a plateau level. This study showed that the decrease in intracellular drug accumulation with time at low drug concentration was due to an increase in cell number as cell proliferation continued (i.e., cell number increased by 36 and 23% over 24 h at 1 and 10 nM, respectively, see Table 1), whereas the increase in drug accumulation with time at high drug concentration was due to paclitaxel-induced increases in total tubulin (Table 4).

Comparison of Paclitaxel Accumulation Data in MCF7 Cells with Previous Data in Other Cells. To determine whether the intracellular pharmacokinetic model is applicable to human cancer cells in general or only to the MCF7 cells, we compared the accumulation of paclitaxel and
drug-induced changes in tubulin polymerization and production in MCF7 cells with the results of an earlier study in HeLa cells (Jordan et al., 1993). The unusually high drug accumulation in MCF7 cells is similar to the finding in HeLa cells; the intracellular-to-initial medium concentration ratios in MCF7 cells, attained at 10 to 1000 nM initial medium concentrations, ranged from 115 to 464 at 24 h (calculated from data in Table 1), whereas the same ratios in HeLa cells ranged from 111 to 480 at 20 h. In both cell lines, prolonged treatment (20–24 h) with paclitaxel at higher concentrations (≥100 nM) induced tubulin production and polymerization and increased microtubule concentration. The enhancement in microtubule concentration in MCF7 cells was lower than in HeLa cells, i.e., a 2-fold enhancement at an initial extracellular concentration of 1000 nM in MCF7 cells versus a 5-fold enhancement in HeLa cells. This 2.5-fold greater enhancement is mainly due to the higher concentration of pre-existing free tubulin available for polymerization in the HeLa cells; free tubulin represents 67% of total tubulin in HeLa cells (Jordan et al., 1991; Thrower et al., 1991) and 25% in MCF7 cells (Table 4). Correction for this factor showed that the enhancement in total tubulin was almost identical in the two cell lines, i.e., 1.8-fold in MCF7 for 24 h versus 1.7-fold in HeLa cells for 20 h (calculated from the literature data; Jordan et al., 1991, 1993; Thrower et al., 1991).

We also compared the binding of paclitaxel in MCF7 cells with the results in J774.2 cells (Manfredi et al., 1982). This earlier study reported an apparent dissociation constant of 80 nM for intracellular binding in J774.2 cells; this value was calculated on the basis of total rather than unbound drug concentration in culture medium. When corrected for the 90% binding to proteins contained in 20% serum added to the culture medium (Song et al., 1996), the dissociation constant in J774.2 cells was calculated to be 8 nM, which is comparable with the 5 nM value in MCF7 cells. These comparisons indicate remarkably similar properties of intracellular paclitaxel binding in human cancer cells, i.e., a) extensive drug accumulation in cells due to binding to intracellular components, b) existence of intracellular saturable and nonsaturable binding sites, c) concentration-dependent induction of tubulin polymerization and production by paclitaxel, and d) comparable binding affinity to intracellular macromolecules. Hence, we propose that the intracellular pharmacokinetic model developed using the MCF7 cells is applicable to other cells, with the following limitations.

The model was developed for paclitaxel accumulation in monolayer cultures of cells that have negligible Pgp expression. For cells where Pgp-mediated efflux contributes significantly to the total efflux of paclitaxel, the kinetic model needs to be refined to include an active drug efflux component. Preliminary results in our laboratory showed that the computational intracellular pharmacokinetic model can be expanded to accommodate the Pgp-mediated efflux in cells transfected with mdr1 (Jang et al., 1998). Drug accumulation in multilayered structures such as a solid tumor needs to take into account the slow and limited drug diffusion, as shown in our recent publication (Kuh et al., 1999). Changes in cell number due to drug treatment depend on the chemosensitivity, which may be cell type-specific and is determined by biological parameters such as the expression of apoptotic and antiapoptotic proteins. Although the increase in microtubule mass may also be cell type-specific, the almost identical values of the paclitaxel-induced tubulin production in MCF7 and HeLa cells suggest otherwise.

**Conclusions.** The intracellular pharmacokinetic model of paclitaxel described here takes into account the known determinants of drug accumulation in Pgp-negative cells, and therefore can be used to depict intracellular drug concentration-time profiles. As shown in this study, a computational intracellular pharmacokinetic model has the versatility to predict the kinetics of paclitaxel uptake, binding, and efflux in cells under various conditions. The ability to predict intracellular drug concentrations as a function of extracellular drug concentrations such as those in plasma enables the comparison of intracellular concentrations attained at different treatment schedules, e.g., a long infusion that delivers a low plasma concentration for a long duration versus a shorter infusion that delivers a higher plasma concentration for a shorter duration. The ability to predict intracellular drug accumulation as a function of extracellular drug binding enables the evaluation of drug-drug interaction due to alteration of free fraction of paclitaxel in plasma by the presence of Cremophor or cisplatin. The ability to relate the intracellular bound concentrations to changes in microtubules enables us to quantify the effects of changes in tubulins on the kinetics of drug accumulation and efflux in cells. Our long-term goal is to develop a model that links the intracellular pharmacokinetics with pharmacodynamics and allows the antitumor effects of selected treatment schedules to be depicted.

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Send reprint requests to: Dr. Jessie L.-S. Au, College of Pharmacy, 500 West 12th Ave., Columbus, OH 43210. E-mail: au.1@osu.edu