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Quantitative Modeling of the Dynamics and Intracellular Trafficking of Far-Red Light-Activatable Prodrugs: Implications in Stimuli-Responsive Drug Delivery System

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Abstract

The combination of photodynamic therapy (PDT) with anti-tumor agents is a complimentary strategy to treat local cancers. We developed a unique photosensitizer (PS)-conjugated paclitaxel (PTX) prodrug in which a PS is excited by near-infrared wavelength light to site-specifically release PTX while generating singlet oxygen (SO) to effectively kill cancer cells with both PTX and SO. The aim of the present study was to identify the determinants influencing the combined efficacy of this light-activatable prodrug, especially the bystander killing effects from released PTX. Using PS-conjugated PTX as a model system, we developed a quantitative mathematical model describing the intracellular trafficking. Dynamics of the prodrug and the model predictions were verified with experimental data using human cancer cells *in vitro*. The sensitivity analysis suggested that parameters related to extracellular concentration of released PTX, prodrug uptake, target engagement, and target abundance are critical in determining the combined killing efficacy of the prodrug. We found that released PTX cytotoxicity was most sensitive to the retention time of the drug in extracellular space. Modulating drug internalization and conjugating the agents targeted to abundant receptors may provide a new strategy for maximizing the killing capacity of the far-red light-activatable prodrug system. These results provide guidance for the design of the PDT combination study *in vivo* and have implications for other stimuli-responsive drug delivery systems.

Keywords

Photodynamic therapy; combination therapy; bystander killing; Paclitaxel; pharmacokinetics; pharmacodynamics; intracellular PK/PD modeling and simulation

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Introduction

Advanced delivery systems that selectively deliver vehicles to tumors increase intratumoral accumulation of therapeutics, while reducing associated systemic side effects [1], [2]. To enhance the selective delivery, a variety of targeting strategies have been applied to delivery systems that include micelles, liposomes, inorganic/hybrid nanoparticles, exosomes, antibody drug conjugates (ADCs), and prodrugs [3–6]. To further improve the selectivity, cargo can be site-specifically released or activated in tumors by various internal mechanisms, such as acidic pH, enzymes, and high reduction potential, and external stimuli, such as light, high-power ultrasound, heat, and magnetic field [7,6,8]. While some of these approaches resulted in clinically valuable drugs (e.g., Abraxane, ADCs, ThermoDox, NanoTherm AS1), there remain many challenges in translating these advanced strategies into clinical practice [9,6]. Two major challenges of such stimuli-responsive drug delivery systems (DDS) are precise control of release behavior and location targeting [10].

External stimuli-responsive DDSs offer unique advantages over those dependent on internal stimuli. Payloads can be released in a highly spatiotemporally and actively controlled manner [6] [11–13]. To express the desired pharmacological action, the locally released drugs should be kept above effective concentrations for enough time within the target area. Detailed understandings of pharmacokinetics (PK) and pharmacodynamics (PD) of such systems at the sites of action are, therefore, critical to improve therapeutic efficacy of the external stimuli responsive system. Mechanism-based, multiscale PK/PD models that delineate each kinetic step of the drug distribution and dynamic processes [14–16] will greatly facilitate the understanding of the PK/PD of stimuli-responsive systems, which are otherwise difficult to separate or influence the other's antitumor effect in ways that are difficult to control experimentally.

In the present study, we developed a quantitative PK/PD model of the external stimuli-responsive systems to better understand determinants of their drug efficacy. As a model system, we used near-infrared (NIR)-responsive prodrugs (Figure. 1), which utilize the photochemistry of photosensitizers (PS), NIR-absorbing materials that generate singlet oxygen (SO) when illuminated, to trigger the release of chemotherapeutic molecules [17]. For example, silicon phthalocyanine (Pc) is conjugated with paclitaxel (PTX) via an SO-cleavable linker to treat local or regional cancers. Our system provides several therapeutic advantages. First, the generated SO can not only cleave the linker, releasing twofold PTX at the site of illumination, but also induces immediate photodamage to tumors [18], known as photodynamic therapy (PDT). PDT can cause tumor cell death by direct cell killing, tumor vascular shutdown, and/or immune system activation [19,20]. Second, this prodrug system provides a spatiotemporally-controlled release of PTX at the tumor site via visible or NIR light activation, thereby avoiding the off-target effects of the released drug. Third, the released PTX can kill the bystander cells escaping from the PDT damage, a result that is called a “bystander killing” effect. Previously, we demonstrated the bystander effect of this light-activatable prodrug under partial illumination [21]. When half of the area of seeded cancer cells was illuminated, PDT damage only occurred in the illuminated area, because of the short diffusion distance of the generated SO, whereas the addition of our prodrug led to

complete cell killing, including adjacent cells in the non-illuminated area, by the released PTX [21].

The antitumor efficacy of our prodrug results from the combination of PDT and site-specific, locally released PTX. The total damage is influenced by many factors, such as the heterogeneous nature of tumors, permeability of the prodrug throughout tumors, drug payload and release efficiency, PS concentration, and light intensity. We used our developed PK/PD model to evaluate the influence of each factor on the antitumor effects resulting from chemotherapy and PDT damage, and to identify key determinants of overall drug efficacy. The findings from this study will guide the further optimization of prodrug design and, eventually, expansion of our PK/PD model for *in vivo* characterization of the prodrugs. Our understanding about this light-activatable prodrug using the developed PK/PD model will provide insights into stimuli-responsive drug release systems in general.

Methods

Materials

Two PTX prodrugs, Pc-(L-PTX)₂ and Pc-(NCL-PTX)₂ (Fig. 1C), were designed for this study, as previously described [17]. Pc-(L-PTX)₂ is the SO-cleavable PTX prodrug that releases PTX upon light activation. The noncleavable prodrug, Pc-(NCL-PTX)₂, uses an SO-noncleavable linker between Pc and PTX, such that the prodrug does not release PTX even after illumination and exerts only PDT damage. All other chemicals or materials obtained from Aldrich Chemical Co., Fisher Scientific, and VWR were analytical grades.

Human SKOV-3 ovarian cancer cells were obtained from the American Type Culture Collection and used for all *in vitro* experiments. All reagents for cell culture were obtained from Invitrogen (Waltham, MA). Cells were maintained in the culture medium [McCoy's 5A medium supplemented with 10% bovine growth serum (FBS), 50 units/mL penicillin G, 50 µg/mL streptomycin, and 1.0 µg/mL fungizone]. Cells were incubated at 37°C in a 5% CO₂ incubator (Sanyo MCO-18AIC-UV).

Prodrug cellular uptake

SKOV-3 cells (10000/well) were seeded onto 96-well plates and treated with 200, 500, and 1000 nM doses of the prodrugs at 37°C under minimum light. After incubation for 0, 1, 3, 6, 9, 12, and 24 h, the medium was collected to quantify the extracellular concentration with 10x dilution with DMSO. DMSO solution (200 µL) containing 10% of the medium was added to each well to lyse the cells. The cell lysate was used to determine the intracellular concentration. The extracellular and intracellular prodrug concentrations were determined by fluorescence measurement of diluted medium and cell lysate. The prodrug concentration was quantified by its fluorescence intensity using a fluorescence plate reader (SpectraMAX Gemini EM, Molecular Devices) with excitation at 605 nm and emission at 680 nm and bottom reading option. Data were analyzed using SoftMaxPro software version 5.4.1.

Determination of fractions of aggregation and protein binding in the medium

McCoy's 5A medium supplemented with 10% FBS was prepared as test medium. Fraction of aggregation ($F_{\text{aggregate}}$) of the prodrug was estimated based on the reduction of fluorescence of the prodrugs in the media compared with their fluorescence in DMSO at the same concentrations of uptake study (200, 500, and 1000 nM) as: $F_{\text{aggregate}} = [Flu_{\text{DMSO}} - Flu_{\text{media}}]/Flu_{\text{DMSO}}$.

To determine the protein binding, stock solutions of 4 mM Pc-(L-PTX)₂ and Pc-(NCL-PTX)₂ in DMSO were diluted with the test medium to the final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM . PBS solution was used as a control. The ultrafiltration centrifuge tubes (Cat. # 8160, 0.22 μm , Corning) were pretreated with 5% tween 80 in PBS for 5 min. After equilibrating for 30 min with 200 μL PBS in the upper cup, any remaining solution was removed. Then, 600 μL of test solution was transferred to the upper cup and equilibrated for 4 h at room temperature. From the upper cup, 200 μL solution was taken to measure total prodrug concentration (C_{Total}). The tube with remaining solution was centrifuged at 3000 g for 15 min. The filtrates at the bottom cup were transferred to measure the free prodrug concentration (C_{free}). Additionally, an aliquot of 20 μL from the upper and bottom cups was further diluted with 180 μL DMSO to fully disperse any aggregates and quantified for prodrug concentration by determining its fluorescence intensity.

The bound drug concentration was described using a capacity-limited protein binding equation [22]:

$$C_{\text{bound}} = \frac{B_{\text{max}} \cdot C_{\text{free}}}{K_d + C_{\text{free}}} \quad (1)$$

where C_{bound} is the bound prodrug concentration and was calculated by subtracting C_{free} from C_{Total} . B_{max} and K_d represent the maximum binding capacity of the protein and dissociation constant of prodrug binding to protein, which was estimated by fitting to the observed data.

Particle size measurement

The particle size of aggregates could affect their rate of de-aggregation and cellular uptake. The extent of aggregation of Pc-(L-PTX)₂ and Pc-(NCL-PTX)₂ at different concentrations was characterized by measuring their hydrodynamic diameter via dynamic light scattering (Brookhaven Instruments Corporation, Holtsville, NY 11742). The size measurement was carried out at with 200, 500, and 1000 nM concentrations of Pc-(L-PTX)₂ and Pc-(NCL-PTX)₂ in McCoy's 5A medium supplemented with 10% FBS.

PTX cellular uptake

After SKOV-3 cells (10000/well) were seeded onto 96-well plates, PTX was added at concentrations of 10, 100, and 1000 nM. After incubation for 0, 0.5, 1, 3, 6, 12, and 24 h, the medium was collected. The cells were trypsinized and centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the cell pellet was dissolved in RIPA buffer. Cell numbers were counted using a LUNATM Automated Cell Counter (Annandale, VA 22003).

PTX concentrations in the medium and cell lysates were quantitatively measured using a validated LC-MS/MS assay with a lower limit of quantification of 1 ng/mL. Briefly, samples (100 μ L) were extracted from cell lysates using methyl *t*-butyl ether containing the internal standard, docetaxel, before evaporation, resuspension, and injection onto the LC-MS/MS system. PTX was chromatographically separated using a Symmetry Shield RP18 column (2.1 $\times$ 50 mm, 3.5 μ m; Waters, MA), with a gradient elution scheme of 0.1% aqueous formic acid and methanol. Percent methanol increased from 40% to 100% in 7 min, before returning to 40% by the end of the 10-min run at a flow rate of 0.2 mL/min. PTX (m/z 852.8 $\rightarrow$ 568.2) and the internal standard (m/z 806.8 $\rightarrow$ 526.3) were detected and quantified using multiple reaction monitoring on an electrospray ionization triple quadrupole mass spectrometer (Micromass Quattro Premier XE; Waters) in the positive ion mode. Other instrument settings were as follows: capillary voltage of 3500 V, cone voltage of 25 V, source temperature 120°C, desolvation temperature 400°C, and desolvation gas flow (N_2) of 600 L/h.

Intracellular PTX released from Pc-(L-PTX)₂ prodrug by illumination

To determine the intracellular fraction of PTX released from Pc-(L-PTX)₂, 50 nM Pc-(L-PTX)₂ was added to SKOV-3 cells at the density of 1 million cells/petri dish (60 $\times$ 15 mm). After 24-h incubation, the medium containing Pc-(L-PTX)₂ was replaced with fresh medium. The petri dish was then illuminated using a diode laser (690 nm) at 5.6 mW/cm² for 30 min. Because PDT damages cell structure during the illumination, both medium and cell lysate were collected for PTX quantification. Uptake of Pc-(L-PTX)₂ at 24 h under these conditions was measured as previously described. The intracellular release fraction of PTX was calculated as: $F_r = [Total\ PTX\ Amount]/[2 \times Intracellular\ Prodrug\ Amount]$.

Cytotoxicity of prodrugs

The cytotoxicity of Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ with illumination, and PTX was determined at various concentrations, ranging from 10 to 1000 nM. On day 0, SKOV-3 cells (5000 cells/well) were seeded on 96-well plates and then incubated for 24 h. Stock solutions of Pc-(L-PTX)₂, Pc-(NCL-PTX)₂, and PTX in DMSO were diluted with the culture medium. The drug solutions (20 μ L) were added to each well containing cells in culture medium (180 μ L) to make the desired final concentrations. For prodrugs, after 24 h incubation in the dark, the medium was either kept or removed (non-washing versus washing). For washing conditions, the cells were washed with PBS three times and incubated in fresh medium before the illumination. The plates were placed without lids on an orbital shaker (Lab-line, Barnstead International) and were illuminated using a diode laser (690 nm) at 5.6 mW/cm² for 30 min to achieve a light dose of 10 J/cm². Then, the plates were placed back into the incubator for another 72 h. After the incubation, MTT assay was performed as previous described [17].

Model development for cellular kinetics and dynamics of prodrugs

The model structure of the prodrug is depicted in Fig. 2. In brief, when illumination begins, the prodrug generates SO, which directly kills cancer cells (i.e., PDT effect) and at the same time cleaves the linker, releasing PTX (Fig. 2). Any residual cancer cells surviving SO damage are later killed by the released PTX (i.e., bystander killing effect). In the case of the

non-cleavable prodrug, however, the generated SO cannot release PTX and only causes SO-induced cell damage. Thus, the cytotoxic effect from the non-cleavable prodrug is similar to the PDT effect of the cleavable prodrug. Because prodrugs could diffuse throughout the cell during the illumination, no subcellular compartments were introduced in the current model. The definitions of the parameters are listed in Table 1.

Cellular uptake of prodrug—The cellular distribution kinetics of the prodrugs were captured in the extracellular (i.e., cell media) and intracellular compartments (Fig. 2). Due to the high lipophilic nature of the prodrugs, the prodrugs exist in both free monomers and aggregates upon addition to the medium (Table S1). It was assumed that only the free monomer form is internalized into cells. The initial free monomer concentration in the medium (C_{fpm}^0) can be depicted using a quadratic equation from Eq. (1).

$$C_{fpm}^0 = \frac{-(B_{max} + K_d - F_{mono} \cdot D) + \sqrt{(B_{max} + K_d - F_{mono} \cdot D)^2 + 4 \cdot K_d \cdot F_{mono} \cdot D}}{2} \quad (3)$$

F_{mono} is the monomer fraction of total prodrug dose (D), which is experimentally determined (Table S2).

The formed aggregates of the prodrugs can be disaggregated over time, as shown by the time-dependent increase in fluorescence intensity when incubated in the medium at 37°C (Fig. S3). The delayed disaggregation process was described by a chain of transit compartments (h) with a transfer rate constant (k_{tr}) [23].

$$\frac{dC_{fpm}^{disagg}}{dt} = D \cdot (1 - F_{mono}) \cdot k_{tr} \cdot \frac{(k_{tr} \cdot t)^h \cdot e^{-k_{tr} \cdot t}}{\sqrt{2\pi} \cdot h^{h+0.5} \cdot e^{-h}} - k_{tr} \cdot C_{fpm}^{disagg} \quad (4)$$

Thus, the free monomer of Pc-(L-PTX)₂ available for cellular uptake comes from either unbound monomer (C_{fpm}^0) or disaggregation (C_{fpm}^{disagg}).

$$C_{fpm} = C_{fpm}^0 + C_{fpm}^{disagg} \quad (5)$$

The free monomer in the medium (C_{fpm}) can be internalized by endocytosis [24]. The endocytosis-mediated internalization is described as a nonlinear process using parameters of the maximum endocytosis rate (J_{max}) and constant (K_{pm}). The prodrug in the cell (C_{tpc}) is effluxed into the media via a first-order process (k_{out}). The total prodrug concentration in medium (C_{tpm}) and in cells (C_{tpc}) is defined as:

$$\frac{dC_{tpm}}{dt} = \left(k_{out} \cdot C_{tpc} - \frac{J_{max} \cdot C_{fpm}}{K_{pm} + C_{fpm}} \right) \cdot \frac{N}{V_m} - Q \cdot C_{tpm} \quad (6)$$

$$\frac{dC_{tpc}}{dt} = \left(-k_{out} \cdot C_{tpc} + \frac{J_{max} \cdot C_{fpm}}{K_{fpm} + C_{fpm}} \right) \cdot \frac{N}{V_c} \quad (7)$$

where N is the total cell number, V_m is the volume of medium, and V_c is the total cell volume. $\frac{N}{V_c}$ can be further simplified into an average single-cell volume ($V_{one\ cell}$), which was obtained from the literature [25].

Unlike the *in vitro* setting, where the drug concentration in the extracellular compartment (e.g., cell media) remains largely unchanged throughout the study period, the amount of drug in the extracellular space *in vivo* is continuously changing as the prodrug is cleared *via* the tumor vasculature, thereby reducing the available amount of drug for uptake. To mimic the *in vivo* environment of tumor vasculature and to investigate the influence of prodrug clearance from the extracellular space on drug efficacy, we added the rate of prodrug removal process (Q) from the extracellular compartment to describe the change of total prodrug concentration in the medium when the medium was replaced for the washing experiment.

Prodrug release and released PTX kinetics—During the illumination, the linker of Pc-(L-PTX)₂ is cleaved by SO and two fold of PTX per prodrug is released, whereas no PTX is released from Pc-(NCL-PTX)₂, because of its non-cleavable linker. The illumination was able to cleave the aminoacrylate linker, but did not influence the structure of PTX. In the present study, the illumination lasted for 30 min and a first-order release rate constant (k_r) was used to describe the PTX release, where k_r is set to zero when there is no light illumination. K_r was calculated based on the time-dependent release kinetics of PTX [17]. We assume a 90% release during the 30-min illumination (release efficacy $F_r = 0.9$), because our previous study showed > 90% PTX release [17]. Both prodrugs are stable under dark conditions until 72 h (Fig S4).

The intracellular kinetics of PTX were adopted based on the previous model [25,26], and PTX model parameters were obtained using experimental results from the current study and the reported study (Table 1, Figs. 2 and 4). The PTX model describes following processes: (1) saturable binding of PTX to proteins in the extracellular compartment ($B_{max,m}$, K_{dm}); (2) PTX uptake and efflux by passive diffusion (CL_{ptx}) and Pgp-mediated efflux (J_{maxp} , K_{pgp}); (3) non-specific binding of PTX to cellular components ($NSBx$) and specific tubulin binding of PTX (K_{dc} , $TB_{max,cin}$); (4) time- and concentration-dependent changes in microtubule mass (k_{bmaxc}), and; (5) time- and PTX concentration-dependent changes in cell numbers (k_{cell} , N). The total extra- and intra-cellular PTX concentrations, C_{txm} and C_{txc} , are expressed as:

$$\frac{dC_{txm}}{dt} = \left(CL_{ptx} \cdot C_{fxc} - CL_{ptx} \cdot C_{fxm} + \frac{J_{maxp} \cdot C_{fxc}}{K_{pgp} + C_{fxc}} \right) \cdot \frac{N}{V_m} + 2 \cdot k_r \cdot F_r \cdot C_{tpm} - Q \cdot C_{txm} \quad (8)$$

$$\frac{dC_{txc}}{dt} = \left(-CL_{ptx} \cdot C_{fxc} + CL_{ptx} \cdot C_{fxm} - \frac{J_{maxp} \cdot C_{fxc}}{K_{pgp} + C_{fxc}} \right) \cdot \frac{1}{V_{one\ cell}} - k_{cell} \cdot C_{txc} + 2 \cdot k_r \cdot F_r \cdot C_{tpc} \quad (9)$$

Similarly, as shown in Eq. (6), the released PTX was also removed from extracellular space when the medium was replaced for the washing experiment, and thus the removal rate (Q) was added in Eq. (8).

The free extra- and intra- cellular PTX concentrations, C_{fxm} and C_{fxc} , are calculated as:

$$C_{fxm} = \frac{-(K_{dm} + B_{max,m} - C_{txm}) + \sqrt{(K_{dm} + B_{max,m} - C_{txm})^2 + 4 \cdot K_{dm} \cdot C_{txm}}}{2} \quad (10)$$

$$C_{fxc} = \frac{-A + \sqrt{A^2 + 4 \cdot (1 + NSB_x) \cdot K_{dc} \cdot C_{txc}}}{2 \cdot (1 + NSB_x)} \quad (11)$$

where,

$$A = (1 + NSB_x) \cdot K_{dc} + T B_{max, cin} \cdot (1 + k_{bmaxc} \cdot (T - T_r)) - C_{txc} \quad (12)$$

T_r is the time of the PTX release from the prodrug, which was 24 h in the present study.

PD model for cytotoxicity—The change in the cell number (N) under unperturbed conditions (i.e., cell growth kinetics) is defined using the logistic function with a first-order natural net growth rate (k_g) of SKOV-3 cells and the maximum cell number (N_{ss}) obtained from the literature [27].

$$\frac{dN}{dt} = k_g \cdot N \cdot \left(1 - \frac{N}{N_{ss}} \right) \quad (13)$$

For the non-cleavable prodrug, the direct cell damage caused by the PDT damage was characterized by a second-order cell killing rate constant (k_{ps}) and the intracellular PS concentration during the illumination (PS_{active}), where $k_{ps} = 0$ without illumination. Throughout the study, light dose was kept constant, and we assumed that SO generation was only dependent on PS concentration.

$$\frac{dN}{dt} = k_g \cdot N \cdot \left(1 - \frac{N}{N_{ss}} \right) - k_{ps} \cdot [PS_{active}] \cdot N \quad (14)$$

For the cleavable prodrug (Fig. 2), the cell growth inhibition resulted from both rapid SO generation (k_{ps}) and the release of PTX (k_{max} and IC_{50}). The cytotoxic effect from the

related PTX is dependent on the intracellular PTX concentration bound to tubulin

[$C_{\text{tubulin}} = \frac{TB_{\text{max}} \cdot C_{\text{free}}}{K_{\text{dc}} + C_{\text{free}}}$], k_{max} is the maximum inhibition rate, and IC_{50} is the tubulin-bound PTX concentration producing 50% k_{max} . A modified signal distribution model with four transit compartments ($M1$ - $M4$) and mean transit time (τ) was used for the delayed cytotoxic effect of PTX [28]:

$$\frac{dM1}{dt} = \left(\frac{k_{\text{max}} \cdot C_{\text{tubulin}}}{IC_{50} + C_{\text{tubulin}}} - M1 \right) / \tau \quad (15)$$

$$\frac{dM2}{dt} = (M1 - M2) / \tau \quad (16)$$

$$\frac{dM3}{dt} = (M2 - M3) / \tau \quad (17)$$

$$\frac{dM4}{dt} = (M3 - M4) / \tau \quad (18)$$

Finally, the changes of tumor cells affected by the cleavable prodrug can be defined as:

$$\frac{dN}{dt} = k_g \cdot N \cdot \left(1 - \frac{N}{N_{\text{ss}}} \right) - k_{\text{ps}} \cdot [PS_{\text{active}}] \cdot N - M4 \cdot N \quad (19)$$

Model parameter estimation

The uptake and cytotoxicity data of free PTX were simultaneously fitted to the model to estimate the PK/PD parameters of PTX (Fig. 4). Protein binding and particle size data were used to calculate fraction of monomer (F_{mono}) and estimate B_{max} and K_d . First, cellular uptake data of both Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ were simultaneously fitted to the model to estimate kinetic parameters for prodrugs. Subsequently, the kinetic parameters were fixed and the cytotoxicity data of Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ were used to estimate PD-related parameters. Time profiles of cytotoxicity (up to 120 h) at different treatment doses were simultaneously fitted to the PD model and the data were plotted as %survival vs. dose by comparing the cell survival at the 96-h endpoint relative to control. All parameters are listed in Table 1 with symbols, definitions, values, and sources. All model fittings used the maximum likelihood algorithm in ADAPT 5 (Biomedical Simulations Resource, CA; in supplementary data). A best model was selected based on the Akaike Information Criterion (AIC) comparison, the goodness-of-fit, weighted residual plots, and reliability of parameter estimations. For simulations, Berkeley Madonna (version 8.3.18, University of California at Berkeley, CA) was used.

Sensitivity analysis

Global sensitivity analysis was performed to estimate the sensitivity of the model output to the parameter values and the correlation of parameters. Three indices (active PS concentration during illumination, tubulin-bound PTX concentration after illumination, % cell inhibition) were used to represent parameter influences on $\text{Pc}-(\text{L-PTX})_2$. Data were analyzed and plotted using R package 3.1.2. The sensitivity changes of the model output to the parameters and the correlation between the parameter sensitivity were analyzed using R package FME [29].

Results

Prodrug internalization was mainly mediated by nonlinear endocytosis

We determined the time-dependent extracellular and intracellular concentrations of prodrugs at various concentrations (Fig. 3). The overall amount of both prodrugs internalized was low, approximately 10% for $\text{Pc}-(\text{NCL-PTX})_2$ and 23% for $\text{Pc}-(\text{L-PTX})_2$. The intracellular drug concentration of $\text{Pc}-(\text{L-PTX})_2$ at 24 h was 1.5–2.5 fold higher than $\text{Pc}-(\text{NCL-PTX})_2$. In the present study, we assumed that only free monomer can be internalized into cells. One possible reason for the different uptake may be the different extent of unbound monomer in the medium, which controls the total amount of prodrug available for internalization. To test this hypothesis, we determined the extents of protein binding and aggregation. We found that, due to the high lipophilicity of the prodrugs, a large portion of the prodrugs formed aggregates upon addition into the cell medium, and about 20% remained as a monomer form in both prodrugs [$F_{\text{mono}} = 0.21$ for $\text{Pc}-(\text{NCL-PTX})_2$ and 0.2 for $\text{Pc}-(\text{L-PTX})_2$] at 1000 nM (Table S2). $\text{Pc}-(\text{NCL-PTX})_2$ and $\text{Pc}-(\text{L-PTX})_2$ showed similar protein binding patterns with high B_{max} and K_d values (Fig. S2). The ratio of unbound to total monomer (fu) did not significantly differ between two prodrugs within the concentration range (200–1000 nM) used for the uptake study (Fig. S2B). Among the total monomers of $\text{Pc}-(\text{NCL-PTX})_2$ and $\text{Pc}-(\text{L-PTX})_2$, ~83% existed in a protein-bound form and ~17% as a free monomer (Fig. S2A). These results indicated that the extent of free monomers was similar for both prodrugs, and protein binding was not the major cause of higher cellular accumulation of $\text{Pc}-(\text{L-PTX})_2$.

Another possible explanation was the different extent of disaggregation. By measuring the time-dependent fluorescence intensity changes, we found that there was a threefold increase in fluorescence intensity of $\text{Pc}-(\text{L-PTX})_2$ from 9 to 24 h, while there was only 1.3 fold increase in $\text{Pc}-(\text{NCL-PTX})_2$ (Fig. S3). The increased fluorescence intensity indicated an increase in a monomer form, caused by the disaggregation process [30]. More $\text{Pc}-(\text{L-PTX})_2$ was reversed to monomers from the aggregates at measurement times after 9 h, compared to noncleavable prodrug $\text{Pc}-(\text{NCL-PTX})_2$. The increased monomer in $\text{Pc}-(\text{L-PTX})_2$ explained, at least in part, why there was a 1.5–2.5 fold higher accumulation at 24 h.

The intracellular kinetic model (Fig. 2) was used to characterize all experimental data from the uptake studies. The mechanisms described for prodrug internalization involve endocytosis or pinocytosis. We used an energy-dependent uptake process (K_{pm} and J_{max}) to determine the internalization of the prodrugs. Our model depicted well the observed

concentration-time profiles of Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ at various concentrations (Fig. 3). The PK parameters estimated for each prodrug are listed in Table 1. The maximum uptake rate (J_{max}) was set equal for both Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂, while K_{pm} values were different. As the dose increased, both prodrugs demonstrated a nonlinear increase of intracellular accumulation. The K_{pm} value of Pc-(L-PTX)₂ was much smaller than Pc-(NCL-PTX)₂ (98 vs. 267 nM). The faster in and out rate of Pc-(L-PTX)₂ maintained the similar uptake amount up to 8 h with Pc-(NCL-PTX)₂. The higher Pc-(L-PTX)₂ accumulation appeared to be mostly attributable to the increase in extracellular disaggregation.

Model validation in prodrug uptake and released PTX fraction

As the concentration range used for cytotoxicity (10 – 200 nM) is lower than the range used for the uptake kinetics (100 – 1000 nM), the amounts of intracellular Pc-(L-PTX)₂ and released PTX at the low concentration of 50 nM were determined and compared with model-predicted values. The experimental data and our model predictions (Table 2) were in good agreement for the intracellular prodrug uptake (15.3. vs 13.6 pmol) and the released PTX (27.7 vs. 29.2 pmol), confirming robust model performance over a wide concentration range. The calculated PTX release efficiency of prodrug was 89%, which further supports our model assumption of 90%, and is close to our previously reported value in medium (93%) [17].

Cytotoxicity and bystander effects from the released PTX

The cytotoxicity profiles of the prodrugs after illumination are shown in Fig. 5A. Once illuminated without washing, both prodrugs showed cytotoxicity with IC₅₀ of Pc-(L-PTX)₂ and Pc-(NCL-PTX)₂ at 3.9 and 24 nM of the extracellular concentrations, respectively. Pc-(L-PTX)₂ showed much stronger cytotoxicity than Pc-(NCL-PTX)₂, since Pc-(NCL-PTX)₂ exhibited only PDT effects, while Pc-(L-PTX)₂ displayed effects of PDT and released PTX combined. The observed cytotoxicity data were adequately characterized by the proposed PK/PD model, except for a slight overestimation of the effect of 100 nM Pc-(NCL-PTX)₂ on SKOV-3 cells.

Both extracellular and intracellular Pc-(L-PTX)₂ can release PTX after illumination. The interchange of the released PTX between cell and medium occurred through passive diffusion or P-gp-mediated efflux (Fig. 2). To demonstrate whether the released PTX in the extracellular space (i.e., the culture medium) could influence cytotoxicity, we evaluated cell growth inhibition with two different procedures (washing vs. without washing, Fig. 5A). Under the washing conditions, we replaced the medium with drug-free fresh medium immediately after 30-min illumination (Fig. 5A). After replacing the medium (dotted lines), the cytotoxicity of Pc-(NCL-PTX)₂ was not significantly changed, because PDT damage occurs mostly due to PSs in the cells and during the illumination. However, the cytotoxicity of Pc-(L-PTX)₂ was largely diminished by replacing the medium. The cytotoxicity profile of Pc-(L-PTX)₂ after washing was close to that of Pc-(NCL-PTX)₂ without washing. This finding suggests that extracellular PTX can significantly influence the cytotoxic effect of PTX, causing bystander cell killing. Through model simulation, we observed a significant change in PTX-tubulin binding profiles, with or without washing (Fig. 5C), but no difference

in PS_{active} (Fig. 5B). This finding shows that the diminished PTX effect was caused by rapid removal of PTX from the intracellular to the extracellular space after washing. The PTX remaining in the medium serves as a reservoir to maintain the prolonged exposure time of tubulin-bound PTX. The retention time of PTX in the extracellular compartment could be important for the bystander effects of $Pc-(L-PTX)_2$. Fig. 5D shows the model simulations of time-dependent cytotoxic effects from 50 nM prodrugs and an equivalent concentration of PTX to the released PTX from the 50-nM cleavable prodrug. $Pc-(NCL-PTX)_2$ only causes transient cell damage during illumination, which is associated with PS_{active} generation. $Pc-(L-PTX)_2$ exhibits transient and sustained cell inhibition by both PS_{active} generation and released PTX.

Efficient cellular uptake and longer retention are important for cytotoxic effects of the prodrug

To better understand how each kinetic step affects cytotoxic efficacy (total cytotoxicity = PDT + PTX effects), we performed a global sensitivity analysis using the developed model (Fig. 6A). We classified the parameters into those related to prodrug, released PTX, and extracellular drug removal. Overall, the cytotoxic efficacy was more sensitive to the kinetics of prodrug than those of PTX, and the PDT effect was influenced more by the prodrug kinetics than by PTX. Protein binding of prodrug in the medium and cellular uptake process were most sensitive to cytotoxicity of $Pc-(L-PTX)_2$. Higher protein binding lowers cytotoxic efficacy, particularly PDT effects, presumably because the protein-bound prodrugs are not taken up by the cells. Interestingly, time-dependent changes in parameter sensitivity (Fig. S5) showed that prodrug protein binding in medium (K_d and B_{max}) could also contribute positively to cytotoxicity during illumination. This may be due to competitive distribution of the prodrug among protein binding, aggregation, and free monomer. More protein binding could reduce free monomer availability, but also prevent the prodrug from being aggregated. Effective endocytosis can further increase intracellular concentrations of prodrug and, thus, cytotoxicity. Among the kinetics of the released PTX, binding and dissociation of PTX from tubulin was the most important process in determining the PTX-induced cytotoxicity. Higher binding affinity ($TB_{max, cin}$) with lower dissociation rate (K_{dc}) can significantly improve the bystander effect under washing conditions (Fig. 6C).

We also investigated how PTX retention, due to washing (Fig. 6B), influenced the cytotoxic efficacy. Q represents the removal rate constant of the prodrug from the extracellular medium. By changing it from 0 to 10 hr^{-1} , the tubulin-bound PTX concentration dramatically decreased, leading to reduced cytotoxicity (Fig. 6B). Q can convert to retention half-life ($TR_{1/2}$). The smaller Q is, the longer the drug can retain. When Q shifted from 10 to 0.1 h^{-1} , $TR_{1/2}$ prolonged from 0.07 to 6.9 h. In the *in vivo* studies, the extent of prodrug retained in tumor site could become the key factor manipulating the bystander effect resulting from the released drug. Calculating the optimal retention time required for drug trapping may be important to exhibit bystander effects from the prodrug system.

Discussion

In the present study, we developed and experimentally confirmed an intracellular PK/PD model of light-activatable, dual-acting prodrug to characterize the cell damage from rapid PDT and sustained cell damage [21] caused by the released PTX at a cellular level. The computational modeling allowed for delineating complex processes (drug distribution and internalization, drug release, and target binding) and identifying the critical factors contributing to the treatment efficacy, when it would not be feasible to isolate the effects of each process experimentally. One of our ultimate goals is to utilize a modeling tool to optimize the design of our stimuli-responsive prodrug system and maximize its therapeutic efficacy *in vivo*.

Effective prodrug internalization improves its anticancer activity, primarily through PDT effect

PDT is a cancer treatment modality that uses visible or near-IR light and photosensitizer to produce reactive oxygen species, mostly singlet oxygen (SO , $^1\text{O}_2$), and induce photodamage to tumor tissues (1). PDT is approved for treatment of several cancers, such as esophageal cancer, non-small cell lung cancer, and bladder cancer (2–5). Based on our sensitivity analysis, most parameters related to prodrug uptake contributed to the PDT damage, and PDT effects were sensitive to prodrug concentrations inside the cancer cells. This is consistent with the fact that direct cell kill by PDT was mostly made by photosensitizers inside cells, as SO has an extremely short lifespan (8) and limited diffusion distance ($\sim 10\text{--}55$ nm) in cells (9). Thus, the internalization of prodrugs to cancer cells from the extracellular space could be critical to achieve the maximum damage *in vivo* via photosensitizers. Formation of aggregates or high protein binding in the medium ($B_{\max}$, K_d) reduces the amount of free drugs available for cellular uptake, which can minimize the cytotoxicity of prodrug, especially on PDT effect. Increased free prodrug in the medium leads to higher cellular uptake. The endocytosis rate parameter ($J_{\max}$, K_{pm}) was the most sensitive parameter that significantly affects cytotoxicity. In this study, the internalization of prodrugs were mediated by non-specific endocytosis, which is commonly influenced by the drug's physico-chemical properties, such as particle size and surface charge [31,32]. Surprisingly, $\text{Pc}-(\text{L-PTX})_2$ and $\text{Pc}-(\text{NCL-PTX})_2$ exhibited highly different endocytosis efficiency, which might be attributable to the differences in the linkers. We speculate that the difference in the linkers (relatively rigid double bond of L vs. flexible single bond of NCL, Fig. 1) might cause this different uptake behavior. The cleavable prodrug with the rigid double bonds could be more rigid than the non-cleavable prodrug, which might make it better in binding to the membrane due to lower entropy. For receptor-mediated endocytosis, the endocytosis rate would largely depend on receptor density.

Retention of released drugs in the target site by vascular shutdown has a great impact on drug efficacy

In the combination of PDT and chemotherapy, direct PDT damage by SO occurs within seconds to minutes, whereas anticancer drugs cause sustained damage over hours to days, killing bystander cells from the PDT damage [33,21]. Effective and complete killing of bystander cells is critical for complete ablation of tumor. Therefore, maximizing the

bystander effect of the released drug is an important step to enhance drug efficacy and reduce tumor recurrence [34,35]. The retention of the released drug within tumors plays a key role for therapeutic outcome. We found that extracellular retention of PTX had the most profound influence on the prodrug efficacy. To mimic the drug clearance through tumor vasculature *in vivo*, we introduced a removal rate constant (Q) to control drug elimination from the extracellular space. The present study revealed that the released PTX cytotoxicity was significantly enhanced by lowering the Q value (Fig. 6B). This suggests that trapping the released drug within tumor via vascular shutdown could be an important strategy for maximizing the efficacy of site-specifically released drugs in tumors (Fig. 1B).

The tumor vasculature is a key target for anticancer therapy and could be also damaged by chemotherapeutic drugs [36,37]. A number of agents have been used to reduce tumor blood flow rate in combination therapy [38,39]. Vascular Disrupting Agents (Tumor-VDA) selectively disrupt the immature and rapidly (>20 times faster than normal) proliferating endothelial cells of established tumor vasculature, either by direct apoptotic effects or by effects related to endothelial cell reliance on a tubulin cytoskeleton to maintain cell shape. These agents can arrest the blood flow in tumors, which leads to cell death in the central part of tumors [40,41]. A VDA is administered with chemotherapeutic agents in a sequence-dependent manner to shut down the intratumoral blood flow. The interval between two drug treatments is very important for drug response [42]. However, a key challenge for limiting tumor blood flow using systemic drug administration is collateral damage to normal tissues [43]. A selective vascular shutdown in tumors is a promising strategy to control the blood flow [44].

The advantage of our prodrug system is its local release of chemotherapy drug during the focused illumination. Only blood vessels in the illuminated tumor area are selectively damaged, which minimizes the damage to collateral arterial blood flow. PDT effects can rapidly cause tumor vessels to shut down [20,21], even during the illumination [45]. Several researchers have reported synergistic antitumor effects from PDT-based combination therapy, with significant vascular damage in tumors [46,47]. In stimuli-responsive systems, an ideal environment for the locally released agents will trap the anticancer drug at the tumor and prolong the cancer cells' exposure to the drug.

Released drug effect is dependent on its effective target binding

Our sensitivity analysis showed that the anti-tumor effect of the released PTX was highly dependent on intracellular drug binding to tubulin ($C_{tubulin}$). Total tubulin density ($TB_{max,cin}$) and the dissociation constant for PTX binding in cells (K_{dc}) were the key factors mediating intracellular accumulation of PTX. Higher efficacy requires both higher drug concentrations and target binding capacity. At high drug concentrations, the target density, rather than drug itself, plays an important role in controlling drug potency [48]. Thus, the target density should be taken into consideration for the selection of chemotherapeutic agents in our PS-conjugate system to achieve better efficacy. Target dissociation rate is another key factor that impacts drug efficacy [49], as slower dissociation increases intracellular accumulation of the released drug. Longer retention of the released drug within cancer cells is critical for effective killing of bystander cells, which can be achieved *in vivo*

through both vascular shutdown and tight target binding. For site-specific drug release systems, anticancer drugs with high affinity and slow target dissociation would be a better choice to improve bystander killing.

Model limitations, application, and future development

Effective intracellular transport of the right concentration of drug molecules to their targets is the major challenge for drug delivery. This is a more critical issue for stimuli-responsive and site-specifically releasing drug delivery systems, because there will be a limited amount of drug released [50]. Our PK/PD model represents a few examples delineating the associated kinetics and dynamics of the stimuli-responsive drug delivery systems. With modifications, the model could be further applied to other stimuli-responsive systems. Similar to our light-response system, others utilize non-invasive methods, such as magnetic waves and ultrasound, to control the drug release [51–53]. Our model can be easily adapted to such external stimuli-responsive systems by changing the parameters associated with the stimuli. Meanwhile, there are many stimuli-responsive systems using the intrinsic stimuli of unique tumor microenvironments (e.g., pH, redox, enzyme, and temperature) [54–56]. In general, drug release controlled with such internal stimuli is harder and slower than drug release prompted by external triggers. For example, the precise control of pH- or temperature-related release could be the limiting step for drug efficacy [57]. By adding the stimuli dependent release process to our model, the ideal release conditions of the carrier could be predicted.

Although our *in vitro* PK/PD model provides some key insights for improving stimuli-responsive drug delivery systems, there are limitations. While we have used a non-specific uptake process in this study, we recently developed light-activatable prodrugs targeted with folate-receptor that may overcome some limitations of the non-targeted prodrugs used here [58]. In addition, we did not consider the effect of light on SO production because the light dose was kept constant in the current experimental settings. Light wavelength, intensity, pulse duration and the distance from the light source are critical factors influencing the effectiveness of PDT [59]. Several researchers have investigated the production, diffusion, and distribution of SO in tumor tissue using the Krogh cylinder model [60,61]. The Krogh cylinder model could be further applied to link the plasma PK of the prodrug and the current *in vitro* PK/PD model [62].

We are currently in the process of expanding our model for the actively targeted drug delivery system. Further, an *in vivo* PK/PD model for stimuli-responsive drug delivery system will be developed to validate our findings *in vitro* and to understand the key antitumor efficacy factors *in vivo*.

Conclusions

In summary, our *in vitro* PK/PD model of the light-responsive prodrug system has provided insights into the kinetic influences on drug efficacy at a cellular level. This system also has implications for *in vivo* systems. The ability to predict treatment outcomes greatly facilitates a better understanding of how PDT combined with chemotherapy may be influenced by the

environment and optimized for maximum efficacy. Our model can be further applied to other stimuli-responsive systems with appropriate minor modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Light-responsive prodrug systems. **(A)** Singlet oxygen (SO)-cleavable prodrug for the unique synergistic combination of PDT and site-specific chemotherapy. **(B)** Efficient bystander killing by the released drugs trapped in the tumor due to the tumor vascular shutdown by PDT. Short-lived SO cannot cause bystander effects, due to its limited diffusion in biological systems. Structures of SO-cleavable PTX prodrug $Pc-(L-PTX)_2$ **(C)**, non-cleavable prodrug $Pc-(NCL-PTX)_2$ **(D)**, and PTX **(E)**.

Figure 2.

Schematic diagram of the intracellular trafficking and dynamic model for light-responsive prodrug. After 24 hr incubation of SKOV-3 cells with Pc-(L-PTX)₂, the cells were illuminated using a 690-nm diode laser ($h\nu$) at 5.6 mW/cm² for 30 min. The model components describing the kinetic and dynamic processes of the released PTX (blue arrows) only apply to the cleavable Pc-(L-PTX)₂.

Figure 3.

The extracellular concentration-time profiles of Pc-(NCL-PTX)_2 (A) and Pc-(L-PTX)_2 (B). The intracellular concentration-time profiles of Pc-(NCL-PTX)_2 (C) and Pc-(L-PTX)_2 (D). Symbols are the experimental data, and lines are model-fitted predictions.

Figure 4.

Time profiles of intracellular PTX concentrations (A) and cytotoxicity (B) at different PTX concentrations in SKOV-3 cells. PTX cellular uptake was determined up to 24 hr.

Cytotoxicity data were collected from literature. SKOV3 cells were incubated with PTX for 3, 6, 12, 24, and 96 hr and MTT assays were performed immediately after incubation.

Figure 5.

(A) The cytotoxicity profiles of Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ in SKOV-3 cells. The experimental data (solid symbols: non-washing; open symbols: washing) were obtained from MTT assays at 72 h after treatment. The lines represent model predictions (solid line: no washing; dashed line: washing). The cell survival curves from 1 to 200 nM were simulated. The % remaining cell numbers relative to control at the 96-hr endpoint were collected and plotted with respect to drug concentration. (B) Model-predicted time profiles of active PS intracellular concentration (PS_{active}), with or without washing. (C) Model-predicted time profiles of tubulin-bound PTX concentrations at IC₅₀ of Pc-(L-PTX)₂, with or without washing. (D) Model-predicted cytotoxicity-time profiles of prodrugs Pc-(NCL-PTX)₂ and Pc-(L-PTX)₂ at IC₅₀ (50 nM) and PTX alone under washing conditions. The

green line represents the simulated cytotoxicity by PTX released from 50 nM prodrug Pc-(L-PTX)₂.

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Figure 6.

(A) Sensitivity of the model output to the parameter values. Positive-sensitivity values indicate that an increase in the parameter will result in an increase in PDT damage, PTX damage, or total cytotoxicity. Negative-sensitivity values indicate that an increase in the parameter will result in a decrease in the outputs. (B) Simulated cytotoxicity profiles of Pc-(L-PTX)₂ at different removal rates. (C) Simulated cytotoxicity profiles of Pc-(L-PTX)₂ with different target binding capacity after washing.

Table 1

PK/PD parameters of the model

Parameters	Definition	Values (%CV)		Source
ICN	Initial cell number	10000		Exp. setting [27]
N_s	Maximum cell number	1×10^8		Exp. setting [26]
V_m (μL)	Volume of cell medium	200		Calculated
$V_{one\ cell}$ (μL)	Volume of a single cell	2×10^{-6}		
k_g (hr^{-1})	Net growth rate constant of cells	0.02803		
Prodrug PK				
		Pc-(NCL-PTX)₂	Pc-(L-PTX)₂	
F_{mono}	Fraction of monomer prodrug in medium	0.21	0.20	Calculated
h	Transit compartment describing delayed disaggregation process	-	6 (68)	Estimation
k_r (hr^{-1})	Disaggregation rate constant	-	0.33 (8.3)	Estimation
B_{max} (nM)	Maximum protein binding capacity in medium	18591 (10)	12042 (4.5)	Estimation
K_d	Dissociation constant of prodrug in medium	5782 (15)	3106 (8.2)	Estimation
J_{max} ($10^{-3} pmol/hr/cell$)	Maximum endocytosis rate from cell membrane	0.40 (31)		Estimation
K_{pm} (nM)	Prodrug concentration producing 50% J_{max}	267 (35)	98 (33)	Estimation
k_{out} ($\mu l/hr^{-1}/cell$)	Outflow rate constant by diffusion	0.5×10^{-6} (33)	2.2×10^{-6} (21)	Estimation
k_r (hr^{-1})	Release rate constant of PTX from prodrug	-	12.6	Calculated
Fr	Fraction of prodrug releasing PTX	-	0.9	[21]
PTX PK				
K_{in} (nM)	Dissociation constants for PTX binding in medium	781		[24]
$B_{max,m}$ (nM)	Maximum PTX binding capacity in medium	3940		[24]
CL_{ptx} ($\mu l/hr/cell$)	Uptake rate constant for free PTX per cell by passive diffusion	0.0129 (16)		Estimation
J_{maxp} ($10^{-3} pmol/hr/cell$)	Maximum efflux rate by p-gp	2.8×10^{-6}		[26]
K_{pgp} (nM)	Dissociation constant of p-gp-mediate efflux	13.9		[26]
K_{dc} (nM)	Dissociation constants for PTX binding in cells	4.93		[24]
NSB_X	Proportionality constant for nonsaturable drug binding in cells	0.148		[24]

Parameters	Definition	Values (%CV)	Source
<i>PD</i>			
k_{ps} (hr^{-1})	Second-order cell kill rate constant of SO	1.95×10^{-5} (10.5)	Estimation
$TB_{max, cin}$ (mM)	Maximum tubulin density at time=0	59200	[24]
K_{tmaxc} (hr^{-1})	Rate constant for changes in maximum tubulin density in cells	0.0108	[24]
τ (hr)	Mean transit time for cell killing signal by PTX	1.56 (54)	Estimation
k_{max} (hr^{-1})	Maximum cell kill rate constant by PTX	0.054 (31)	Estimation
IC_{50} (nM)	Tubulin-bound PTX concentration producing 50% k_{max}	10115 (67)	Estimation

Table 2

The observed and model-predicted values of prodrug uptake and released PTX

	Observed	Model-Predicted
Pc-(L-PTX) ₂ cell uptake (pmol)	15.3 ± 1.1	13.60
Released PTX (pmol)	27.4 ± 6.1	29.20