SUPPLEMENTARY INFORMATION

Synergistic Targeting of Cell Membrane, Cytoplasm and Nucleus of Cancer Cells using Rod-Shaped Nanoparticles

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SI Text 1. Fluorescence intensity of CPT: Since CPT is a fluorescent molecule, it is possible that the fluorescence of CPT can be self-quenched due to resonance energy transfer between CPT molecules. It is well known for many fluorescent molecules such as FITC which is self-quenched when bound to human serum albumin proteins.[1] Anti-cancer drug doxorubicin is also self-quenched at concentrations ≥25μg/ml.[2] We have not tested the self-quenching behavior of CPT in its compact nanoparticle form in the scope of this study which is essential to measure intracellular CPT concentrations directly within the cells. However, all the CPT concentrations reported in this study were measured from a linear standard curve of CPT nanorods of 0-100μg/ml in PBS, which did not suppress the quantum yield as expected to observe in its concentrated crystal form. In this study, we did not measure CPT concentrations within the cells from its fluorescence intensity.

SI Text 2. Colocalization analysis of CPT nanorods and TTZ: A colocalization analysis between Alexa 594-TTZ and CPT nanorods shows a high overlap coefficient, R=0.73 demonstrating 73% CPT nanorods colocalized with 73% TTZ in the cytoplasm after 2 h (**Table I**). The Pearson's correlation coefficient, R_r = 0.7 represents that there is a positive correlation between red (TTZ) and blue (CPT) signals (**Table I**). This number matches well with the colocalization coefficients, m1 and m2, showing an overlap of 63% red intensity with 99% blue intensity (**Table I**). A longer incubation of CPT-TTZ to 24 h leads to decreased colocalization of CPT and TTZ. After 24h, percent colocalization (R=0.44) between CPT and TTZ was decreased by 39.7% compared to that at 2 h (R=0.73) (**Table I**). Both red (m1 = 0.58) and blue (m2 = 0.62) colocalization coefficients decrease after 24 h. The correlation (R_r = 0.2) dropped to one-third of that at 2 h (R_r = 0.7). This indicates the dissociation of TTZ from CPT nanorods after 24 h, and translocation of TTZ and CPT nanorods separately into different sites of the cell.

SI Text 3. Colocalization analysis of TTZ and transferrin: To determine if TTZ is internalized into sorting endosomes and recycled back to the plasma membrane, we investigated the colocalization of CPT-TTZ with Alexa 488-conjugated transferrin which is a known endosomal recycling marker (**Fig. 3**). The two ligands, TTZ and transferrin showed 77% colocalization (**Table II**; R=0.77 for red:green channels) indicating that almost all the TTZ that were attached to CPT nanorods (R=0.73; **Table I**) resided in the recycling endosomes. The overlap between Alexa 594 TTZ and transferrin consists of 72% (m1) red intensity and 84% (m2) green intensity, in which the red signal intensity increases by a factor of only 0.44 (Rr=0.44 for red:green channels) if the green intensity increases (**Table II**). This moderate positive correlation between red (TTZ) and green (transferrin) signals indicates that TTZ localization is not affected much by transferrin relocalization in case transferrin undergoes any lysosomal degradation or increases its intracellular transport.

SI Text 4. The median effect doses and shape coefficients of the dose-effect relationships for each drug in CPT-TTZ-DOX: The Chou-Talalay analysis[3] is based on the median effect equation, which states that: $\frac{f_a}{f_u} = (\frac{D}{D_m})^m$ (1)

where f_a and f_u are respectively the fractions of the affected and unaffected systems by the dose D. D_m is the dose required to produce the median effect (IC_{50}) and m is the parameter that dictates the shape of the dose-effect curve. Eq. (1) can be rewritten as follows:

$$\log(\frac{f_a}{1-f_a}) = m\log D - m\log D_m \qquad (2)$$

A linear plot of $\log(\frac{f_a}{1-f_a})$ *vs.* $\log D$ was drawn for each drug to calculate the two

parameters *m*, and theoretical IC_{50} value D_m from the slope and intercept, respectively. The plots for all three drugs (CPT, TTZ, DOX) have been added to **SI Fig. 8e-f**. The shape coefficients (*m*) of the dose effect curves of TTZ, CPT and DOX, and the median effect doses (D_m) and have been included in **SI Table III**. The *m* values of all three drugs are <1, and also differ from each other which indicates that the drugs are mutually nonexclusive and, thus, have different mechanisms of inducing cell death in BT-474 cells, and differences in kinetic orders to induce cytotoxicity. TTZ showed shallow dose-effect curve (**SI Fig. 8d**) and a lower *m* value than CPT and DOX (**SI Fig. 8e and 8f**) indicating that BT-474 cells are less sensitive to TTZ than CPT and DOX.

The D_m values indicate different potency of the drugs; however, the values (**SI Table III**) are higher than the experimental IC_{50} values (**SI Fig. 8a-8c**) perhaps because of insensitivity of BT-474 cell growth inhibition above 50%, especially for TTZ and CPT treatments (**SI Fig. 8a and 8b**). However, in combination treatment, IC_{50} of the drugs using CPT-TTZ-DOX were 10-10,000 fold lower than both theoretical D_m values and experimental IC_{50} values with a CI<1, suggesting a synergistic combination effect compared to the individual drug treatment.

SI Text 5. Preparation of polystyrene-TTZ nanoparticles: We used rod-shaped polystyrene nanoparticles in the cell cycle analysis experiments as a control to show TTZ activity without nanoparticle cytotoxicity. We prepared the polystyrene nanorods from spherical nanoparticles using our previously established method.[4] Briefly, we trapped spherical nanoparticles in 10% polyvinyl alcohol (PVA) containing films prepared with 0.5% (wt/vol) glycerol. The spherical particles in films were stretched in one direction at ~120°C, extracted from the films by dissolving in 30% isopropanol-water mixture, and washed with a gradient (20, 10, 5 and 0%) of isopropanol-water. Using an aspect ratio 3, nanorods of 440.3 ± 74.2 nm were prepared. The

nanorod surfaces were coated with TTZ antibody using physical adsorption method as a nanorod:antibody weight ratio of 8.3. An SEM image of polystyrene-TTZ nanoparticles is shown in **SI Fig. 9(a)**.

SI Text 6. Preparation of 290 × 47.4nm CPT nanorods: The size of CPT nanorods can be further reduced for *in vivo* studies. To obtain smaller size nanoparticles, we modified the solvent-diffusion method used in this study. Briefly, 2 ml of CPT-DMSO solution (1 mg/ml) was added to 20 ml of pH 3.5 water under continuous stirring (1000 rpm) and sonication at room temperature. The samples were stirred and sonicated for additional one hour, and washed three times using pH 3.5 water. An SEM image of these particles is shown in **SI Fig. 11**.

SUPPLEMENTARY FIGURE LEGENDS

SI Figure 1: Stability of CPT nanorods after 72 h incubation in serum-containing medium at pH 7.4, 6.0 and 4.5. Scale bar = 500nm

SI Figure 2: Release profiles of DOX from CPT-TTZ-DOX nanoparticles in 10% FBS containing PBS. Data is shown as mean ± S.D. (n=3)

SI Figure 3: Z stacks of confocal sections (1μm) with the overlay of CPT (blue) and Alexa 594 TTZ (red) color channels to show the intracellular distributions of CPT and TTZ in BT-474 live cells after **(a)** 2 h and **(b)** 24 h incubations. **(a)** Magenta colors correspond to significant colocalization between majority of the CPT and TTZ in the middle sections of the cells after 2 h. **(b)** After 24 h, most of the TTZ recycles back to the plasma membrane as seen from the red signals from top to bottom z stacks. CPT resides inside the cells as revealed from the blue signals from the middle of the z sections to the bottom of the cells.

SI Figure 4: Fluorescence confocal microscopic images of the uptake of **(a)** CPT nanorods control, **(b)** CPT-Alexa 594 TTZ after incubating cells with an excess of TTZ solution for 1 h, and **(c)** a drug cocktail of soluble CPT in DMSO, TTZ (or Alexa 594 TTZ) solution in PBS and free DOX by BT-474 cells. Cell nuclei were stained using SYTO 13 dye while imaging Alexa 594 TTZ. BT-474 cells were incubated with the nanoparticles for 2 h at 37°C prior to live cell imaging after 24 h. In **(a)** and **(b)**, no blue (CPT) or red (Alexa 594 TTZ) fluorescence signals were detected in BT-474 cells indicating the inability of CPT internalization without the TTZ-specific interactions. **(c)** DMSO soluble CPT precipitated out of the cells and could not enter the cells either. Free DOX entered the cell nuclei, however, TTZ could not recycle back to the plasma membrane indicating the role of nanoparticle size and shape for efficient intracellular distribution of drugs to exert their therapeutic activity.

SI Figure 5: Average fluorescence intensity of Alexa 594 conjugated TTZ per cell was measured from confocal microscopic images using ImageJ and Imaris. BT-474 cells were treated with CPT-Alexa 594 TTZ nanorods for 2 h and imaged after 2 and 24 h using confocal microscopy. Three independent microscopic images and thirty cells were analyzed to measure the average fluorescence intensity.

SI Figure 6: Subcellular localization of Alexa 594 conjugated anti-human IgG (red channel) added as CPT nanorods into BT-474 cells. BT-474 cells were treated with CPT-Alexa 594 IgG nanoparticles for 2 h and imaged live after 24 h. Unlike TTZ, IgG control is localized in single spots in the cytoplasm without recycling to the plasma membrane. No red signal is detected at the cell surface. Magenta color is the overlay of red IgG and blue CPT.

SI Figure 7: TEM images of **(a)-(b)** BT-474 cells with internalized CPT-TTZ nanoparticles and **(c)** PBS-treated BT-474 cells (negative control) that did not show any nanoparticle inside the cell. **(a)-(b)** Clusters of CPT-TTZ nanoparticles were found in the endocytic vesicles of these cells as indicated by the arrows.

SI Figure 8: Dose response curves of the single agent treatment of **(a)** CPT, **(b)** TTZ and **(c)** DOX, and their corresponding median effect plots (**(d)** CPT, **(e)** TTZ and **(f)** DOX) in BT-474 cells. Cells (10,000/0.2ml/well) were incubated with CPT and TTZ for 72 h in 96-well plates and analyzed using calcein-AM. For DOX treatment, cells were incubated with DOX for 2 h, re-incubated in fresh medium for 72 h and analyzed. The percentage growth inhibition is expressed as a percentage of control cells with PBS treatments. The average values and S.D. represent four replicates from at least two independent experiments. A linear regression **(d)-(f)** to experimental data of **(a)-(c)** was applied to obtain estimates of the two parameters D_m and m. The values are shown in **SI Table III**.

SI Figure 9: (a) SEM image of polystyrene nanoparticles. Scale bar = 500nm, **(b)** Cell growth inhibition of MDA-MB-231 control cells treated with CPT-TTZ-DOX and CPT-BSA-DOX nanoparticles. There is no significant difference in growth suppression between these nanoparticles in Her2 negative MDA-MB-231 cells. The results indicate active targeting of Her2 receptors in BT-474 cells by TTZ and thus higher efficacy in combination with CPT nanorods and DOX.

SI Figure 10: Cell cycle analysis graphs of BT-474 cells after treated with TTZ alone, TTZ delivered with polystyrene nanorods (positive control), BSA coated polystyrene nanorods (negative control), CPT-TTZ and CPT-TTZ-DOX. The effects of the drugs on cell cycle progression were evaluated using Vybrant DyeCycle Violet stain (Invitrogen) and flow cytomtery.

SI Figure 11: SEM image of 290±88 × 47.4±19.1 nm CPT nanorods. Scale bar = 1 μm.

SUPPLEMENTARY TABLES

SI Table I: Quantification of TTZ, BSA and DOX loading on CPT and CPT-TTZ nanoparticles as determined using micro-BCA protein assay kit and DOX fluorescence standard curve. The zeta potential of the nanoparticles in PBS were measured using a Nanoseries Zetasizer (Malvern).

SI Table II: Statistical significance of CPT-TTZ-DOX treatments using Student's t-test compared to other treatments. The combination treatment using TTZ-CPT-DOX shows significantly different with p values ≤ 0.05 compared with the control treatments.

SI Table III: The values of D_m and m for CPT, TTZ and DOX as calculated from the median effect plots as shown in **SI Fig. 8e-8f**.

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