Supporting Information

Disulfide Bond Bridge Insertion Turns Hydrophobic Anticancer Prodrugs into Self-Assembled Nanomedicines

Yongjun Wang,†‡*, Dan Liu,§‡, Qingchuan Zheng,‖ Qiang Zhao,‖ Hongjuan Zhang,‡ Yan Ma,‡
John K. Fallon,† Qiang Fu,‡ Matthew T. Haynes,† Guimei Lin,‡ Rong Zhang,‡ Dun Wang,§
Xinggang Yang,‡ Linxiang Zhao,§ Zhonggui He,‡,* and Feng Liu,†,§

†Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill 27599, USA
‡School of Pharmacy, and §Key Laboratory of Structure-Based Drug Design and Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China
‖ State Key Laboratory of Theoretical & Computational Chemistry, Institute of Theoretical Chemistry, Jilin University, Changchun 130012, China
§ School of Chemical Engineering, Sichuan University, Chengdu 610065, China
#, Department of Medicinal Chemistry, School of Pharmacy, Nanjing Medical University, Nanjing 210029, China
§ School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou 510405, China
§ School of Pharmacy, Shandong University, Jinan 250012, China
§ School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China

* Corresponding authors: (Z.H.) hezhonggui@syphu.edu.cn; (Y.W.) wangyjspu@163.com

‡ Deceased

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Dedicated to the memory of Professor Feng Liu, PhD, 1955-2014
University of North Carolina at Chapel Hill
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Materials and Methods

Preparation and characterization of DSINMs. DSINMs were prepared by nano-precipitation. Briefly, the tested compound was dissolved in ethanol and then added dropwise to water, under mechanical stirring (~600-800 rpm) at room temperature, to give a final ethanol concentration of 2-5 % ethanol. Under these conditions, the self-assembly of DSINMs occurred spontaneously. The ethanol could also be replaced with DMSO which is an FDA approved agent (Rimso-50) for bladder instillation. PEGylated DSINMs were produced by mixing DSINMs with DSPE-PEG2000 in water (DSPE-PEG2000/DSINMs = 15/100, w/w). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to determine particle size and morphology. TEM images of all samples were acquired using a JEOL 100CX II TEM (JEOL USA, Inc., Peabody, MA). Tested samples (5 µL) were dropped onto a 200 mesh carbon-coated copper grid (Ted Pella, Inc., Redding, CA) and allowed to stand for 5 min. Following this, the excess liquid was wicked off. Grids were then stained with 1 % uranyl acetate (5 µL) for 10-20 sec and wicked dry. All images were acquired at an accelerating voltage of 100 kV. Scanning electron microscope (SEM) was performed using an INCA Penta-FET Energy Dispersive Spectroscopy (EDS) System (Oxford Instruments, Abingdon, UK) attached to a Hitachi S-4700 FE-SEM (Hitachi, Ltd., Tokyo, Japan). SEM images of the nanoparticles were taken on glass substrate. A Cressington 108 Auto Sputter Coater equipped with an Au/Pd (80/20) target and MTM-10 Thickness Monitor (Cressington Scientific Instruments Ltd., Watford, UK) was used to coat the sample with a conductive layer before taking SEM images.

Separation of DSINMs by sucrose density gradient centrifugation (SDGC). 0.9 mL of sucrose solution was sequentially overlaid on a thin ultracentrifuge tube to form a 60-40–20–0 % discontinuous gradient from bottom to top. 100 µl samples of DSINMs containing different prodrugs, in water, were added at the top. The gradients were centrifuged using a Beckman Coulter SW 60Ti rotor (Brea, CA) at 50,000 rpm for 2.5 h (20 °C). Then, fractions (0.9 mL each) were collected from top to bottom and transferred into glass tubes to detect the visible scattering of light
along the path of a laser beam. 5-FU-S-S-VE and DOX-S-S-VE were measured using HPLC and fluorescence spectrometry, respectively.

**HDACs inhibition assay**

HDACs were prepared from HeLa cell extracts. Compounds were initially screened at final concentrations of 30 nM using an enzymatic assay measuring total HDACs activity.

*In vitro* HDACs inhibition assays were conducted using the HDAC Colorimetric Activity Assay/Drug Discovery Kit (BML-AK-501, Enzo Life Sciences, Inc., Farmingdale, NY) according to the manufacturer’s recommendations[4]. Briefly, 5 µL of HeLa Nuclear Extract was mixed with 10 µL of test compounds, including vorinostat. After 5 min, fluorogenic substrate Boc-Lys (acetyl)-AMC (25 µL) was added, and the mixture was incubated at 37 °C for 15-30 min. The reaction was then stopped by addition of 50 µL of developer containing trypsin. After incubation at 37 °C for 10-15 min, the light emission of deacetylated substrate was quantified at 405 nm. The inhibition ratios were calculated from the fluorescence intensity readings of tested wells relative to those of control wells. Experiments with triplicate data were performed. The IC$_{50}$ values were calculated using a regression analysis of the concentration/inhibition data.

**HDACs cytotoxicity assays**

To test the anticancer activities of the synthesized compounds we evaluated antiproliferative activities of these compounds against human breast cancer cell line MCF-7 and the human promyelocytic leukemia cell line HL-60, using the MTT assay and Trypan blue method, respectively. Both cell lines were purchased from the ATCC (American Type Culture Collection, Rockville, MD, USA) and cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM glutamine and 10 % heat inactivated fetal bovine serum (Gibco).

MCF-7 cells were seeded at a density of $2 \times 10^4$ cells/mL and incubated with various concentrations of the tested compounds for 72 h. The compounds were dissolved in dimethyl sulfoxide (DMSO), with the final DMSO concentration in the samples not exceeding 0.1 %. The
optical density was read at 570 nm using a Tecan microplate reader (Morrisville, NC). Cell viability was determined using the MTT assay. The growth inhibitory ability of the compounds was calculated and expressed as the ratio of the cell number in the treated group to that of the untreated group. The IC\textsubscript{50} (concentration which inhibited half of the cell growth) was calculated.

HL-60 cells in logarithmic growth were seeded at a density of 4.5 × 10\textsuperscript{4} cells/mL and incubated with various concentrations of the test compounds for 72 h. The compounds were dissolved in DMSO and then diluted to the proper concentrations such that the final concentrations of DMSO were less than 0.1 % in the culture medium. Cell viability was determined after staining the cells with Trypan blue. The Trypan blue stained (nonviable) cells and the total cell number were determined using a hematocytometer. The growth inhibition in cells after the treatment with different concentrations of the compounds was calculated by comparing the data with that of control cells. The GI\textsubscript{50} (half-growth inhibitory concentration) was obtained from regression analysis of the concentration response data.

**Molecular dynamics simulations.** A tetramer structure of PTX-S-S-VE for molecular dynamics (MD) simulations was generated by GaussView 5.0.8 (Gaussian, Inc., Wallingford, CT). Antechamber was used to generate parameters of the PTX-S-S-VE for the AMBER and associated GAFF force fields, and the AM1-bcc model was used to generate the atomic charges. The MD simulations, including the energy minimization, were performed by using AMBER 11 software package. The system was solvated with the TIP3P water model in a truncated octahedron box with a 10 Å distance around the solute using xLEAP. The PTX-S-S-VE molecules were fixed with a 50 kcal mol\textsuperscript{-1} Å\textsuperscript{-2} constrain, and solvent was energy minimized for 2,000 steps using the steepest descent (SD) method followed by a further 2,000 steps using conjugate gradient algorithms. Subsequently, these initial harmonic restraints were gradually reduced to zero during energy minimizations. After that, the system was minimized by the SD method and switched to conjugate gradient every 3,000 steps for a total of 6,000 steps without harmonic restraints. Thereafter, the system was gently heated from 10K to 300K, applying harmonic restraints with a force constant of 10 kcal mol\textsuperscript{-1} Å\textsuperscript{-2} on the solute atoms, and then equilibrated for 2,000 ps. Finally, production MD
simulation was carried out for 10 ns to check the self-assembly process. The particle mesh Ewald (PME) summation method was applied to treat the long range electrostatic interactions with a periodic boundary condition. All bonds involving hydrogen atoms were restricted by the SHAKE algorithm. The time step in all MD simulations was 2 fs. PyMOL and VMD software were used to visualize the trajectories and to depict structural representations.

**Pharmacokinetic studies.** Female CD-1 mice (18-22 g) received injections of a dose of 5 mg/kg of Taxol or PEGylated (with DSPE-PEG2000) PTX-S-S-VE DSINMs (PTX equivalent dose) into the tail vein. At 10, 30, 60, 120, 240 and 1,440 min after injection, blood samples were collected from the suborbital vein into heparinized tubes. All samples were immediately centrifuged for 10 min at 5,000 rpm, and the plasma was transferred into clean glass tubes. Then, to 50 µL plasma, 100 µL I.S. solution (2’-(3-methylthio propyl)paclitaxel) and 2 mL methyl tert-butyl ether were added, followed by vortex mixing for 90 sec and centrifugation at 3,500 rpm for 10 min. The upper organic phase was transferred into fresh tubes and evaporated to dryness under a stream of filtered dry air. The samples were then reconstituted with 100 µL of HPLC mobile phase. Aliquots of 20 µL were injected into the HPLC system for analysis. PK results were calculated using a noncompartmental pharmacokinetic model (Win-NonLin software, Certera USA, Inc., St. Louis, MO). Parameters determined for PTX in plasma included mean retention time (MRT), volume of distribution (V<sub>d</sub>), total body clearance (Cl), t<sub>1/2</sub> and AUC.

**In vivo anticancer effect.** Human epidermoid carcinoma cell line KB-3-1 cells (2 ×10<sup>6</sup>) were injected subcutaneously into the flank region of female nude mice for inoculation. Once the tumor mass in the xenograft was established, mice were randomly assigned to treatment groups (5 mice per group). Tumor volume could be calculated from the formula: tumor volume = (W<sup>2</sup> × L)/2, where W and L represent the minor and major axes of the tumor, respectively. The tumor bearing mice were injected via the tail vein with Taxol or PEGylated PTX-S-S-VE DSINMs at PTX equivalent doses of 5 mg/kg on days 0, 2, 4, 6, 8 and 10. Saline was used as a control. Tumor volume and body weight were monitored every three days. Animals were treated in accordance with
National Institute of Health Guide for the Care and Use of Laboratory Animals as approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

**In vitro evaluation of drug induced hemolysis.** Blood was collected from the BALB/c mice and centrifuged at 4 °C. The plasma was removed and erythrocytes were washed twice with PBS. Erythrocytes \((2 \times 10^{10})\) were resuspended in 100 µL of PBS containing either Taxol or PTX-S-S-VE DSINMs at a range of µM PTX concentrations. These procedures were done on ice. After incubation for 60 min at 37 °C, the samples were centrifuged for 4 min at 13,500 rpm. 50 µL supernatants were then diluted with 150 µL PBS and the optical densities (ODs) were measured at 570 nm. The percentage hemolysis was calculated as a function of OD according to the equation: hemolysis % = ODsample/ODtotal hemolysis × 100 %. The OD of total hemolysis was measured using samples that had been sonicated for 5 min before the measurement.

**Tumor imaging.** Female nude mice were implanted subcutaneously with KB-3-1 cells \((5 \times 10^6)\) into the right flank area. Five days after the inoculation free SRB and PEGylated SRB-S-S-VE DSINMs (2 mg SRB/kg and the molar equivalent of SRB-S-S-VE) were intravenously injected through the tail vein of the tumor bearing mice. The mice were imaged under anesthesia several times after injection using the Kodak In-Vivo FXPro Imaging System. All images were captured using the same exposure time at Ex/Em = 550/600 nm.

**Statistical analysis.** Data were expressed as mean ± s.d. Differences between groups were assessed using the paired, two-sided Student t-test. *\(p < 0.05\) was considered significant, and **\(p < 0.01\) was considered highly significant.
Scheme S1: Synthesis of PTX-VE

Paclitaxel (PTX) (100.0 mg, 0.117 mmol) was reacted with d-α-tocopherol succinate (62.0 mg, 0.117 mmol) in the presence of N,N'-dicyclohexylcarbodiimide (48.2 mg, 0.234 mmol) and a catalytic amount of dimethylaminopyridine in anhydrous dichloromethane (8 mL) at room temperature under nitrogen atmosphere for 7 h. The resulting mixture was filtered to remove N,N-dicyclohexylurea and the filtrate was dried under vacuum. The residue was purified using silica gel column chromatography, eluting with a chloroform-methanol solution of gradually increasing methanol content. The elution solvent was removed in a vacuum to give 95.9 mg of PTX-VE (VE = vitamin E) conjugate with the total yield of 60%.
Dithiodiglycolic acid (1) was converted to the corresponding anhydride (2) with acetic anhydride as dehydration agent. Excessive acetic anhydride was removed with toluene under high vacuum at room temperature. The residue was reacted with VE, with DMAP as catalyst, to rapidly obtain the acid 3. This was condensed with PTX under DCC and DMAP to produce the target compound PTX-S-S-VE.

Mass spectral (MS) data was acquired on a Finnigan MAT mass spectrometer (Thermo Fisher Scientific, Waltham, MA) following LC separation (LC–MS). $^1$H NMR spectra were recorded on a Varian Inova 400 MHz spectrometer (Agilent Technologies, Santa Clara, CA) at room temperature in deuterated chloroform (CDCl$_3$) with tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in ppm (δ). All chemicals were obtained from commercial suppliers and used without purification. Column chromatography was performed with silica gel 60 (200-300 mesh).

A solution of dithiodiglycolic acid (1) (0.2 g, 1.10 mmol) and 3 mL anhydrous acetic anhydride was stirred for 2 h at 30 °C. The solution was then evaporated to dryness under high vacuum, with addition of toluene three times. To the residue was added dichloromethane (2 mL), VE (0.1 g, 0.23 mmol) and a catalytic amount of DMAP, stirring at room temperature for 5 min.
The product (compound 3) was purified by silica gel column chromatography, eluting with a solution of hexane, ethyl acetate and acetic acid. The yield was 56.3 %. $^1$H NMR (400 MHz, CDCl$_3$, ppm): $\delta$ 3.87 (2H, s), 3.68 (2H, s), 2.60-2.57 (2H, m), 2.09 (3H, s), 2.04 (3H, s), 2.00 (3H, s), 1.83-1.75 (2H, m), 0.87-0.83 (12H, m).

Acid 3 (0.5 g, 0.84 mmol) and dichloromethane (10 mL) were stirred in an ice-water bath, with addition of DCC (0.20 g, 1.0 mmol) and DMAP (13.8 mg, 0.01 mmol). After 5 min, PTX (0.72 g, 0.84 mmol) was added into the solution. The solution was stirred for a further 2 h, filtered to remove DCU and purified by silica gel column chromatography. The elution solution was chloroform and methanol (200:1) and the product was a white solid with a yield of 60.7 %. Chemical formula C$_{80}$H$_{103}$NO$_{18}$S$_2$. MS (m/z): 1452.3 [M+Na]$^+$, 1453.3 [M+Na+H]$^+$. $^1$H NMR (400 MHz, CDCl$_3$, ppm): $\delta$ 8.15-8.13 (2H, m), 7.75-7.73 (2H, m), 7.61-7.59 (2H, m), 7.54 (2H, m), 7.52-7.17 (7H, m), 6.29-6.25 (1H, m), 6.01-5.99 (1H, m), 5.69-5.67 (1H, m), 5.53-5.52 (1H, m), 4.99-4.96 (1H, m), 4.46-4.45 (1H, m), 4.33-4.30 (1H, m), 4.21-4.19 (1H, m), 4.07 (1H, m), 3.82-3.70 (5H, m), 3.49-3.47 (2H, t), 0.87-0.86 (15H, m).
VE and thiodiglycolic anhydride (4) were reacted under the catalysis of DMAP to obtain the acid 5, in the same way as for the synthesis of compound 3. Acid 5 and PTX were condensed under DCC and DMAP to provide the target compound PTX-S-VE. VE (0.1 g, 0.23 mmol) and thiodiglycolic anhydride (4) (0.91 g, 0.69 mmol) were dissolved in dichloromethane (2 mL), and a catalytic amount of DMAP was added while stirring. After 1 h, the reaction was terminated. Compound 5 was obtained as a white solid by silica gel column chromatography, eluting with a solution of hexane and ethyl acetate and a yield of 67.3 %.

The PTX-S-VE was synthesized using a similar preparation method as that used for PTX-S-S-VE (Scheme 2), the main difference being that acid 3 was replaced by acid 5. The yield was 57.3 %. $^1$H NMR (400 MHz, CDCl$_3$, ppm): $\delta$ 8.15 (2H, d), 7.72 (2H, d), 7.60 (1H, m), 7.51-7.49 (2H, m), 7.37 (4H, m), 7.31-7.26 (4H, m), 6.30 (2H, s), 6.04 (1H, dd), 5.69 (1H, d), 5.50 (1H, d), 4.99-4.96 (1H, m), 4.48-4.43 (1H, m), 4.32-4.30 (1H, d), 4.21-4.19 (1H, d), 3.81-3.83 (1H, d), 3.71-3.68 (1H, d), 3.50-3.40 (4H, m), 2.60 (3H, m), 2.49 (1H, d), 2.42 (3H, s), 2.23 (3H, s), 2.10 (3H, s), 2.03-1.93 (10H, s), 1.70-1.68 (4H, m), 0.88-0.83 (17H, m).
Target compound DOX-S-S-VE was obtained by the coupling reaction of commercial doxorubicin hydrochloride (DOX) and the acid 3 in the presence of o-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU). DOX (100 mg, 0.18 mmol) was suspended in dry dichloromethane (25 mL). Acid (3) (107 mg, 0.18 mmol) and HBTU (230 mg, 0.60 mmol) in dry dichloromethane (20 mL) were added slowly to the reaction mixture. Then, N,N-diisopropylethylamine (DIPEA, 200 mg, 1.54 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 3 h under a nitrogen atmosphere. After confirmation of completion of the reaction using a TLC plate, water (80 mL) was added to the mixture and the crude product was extracted with chloroform (3 x 50 mL). After removal of solvent under reduced pressure, the crude product was purified by silica gel column chromatography, eluting with chloroform/methanol (0-10 % methanol) giving a dark red powder with the yield of 47.6 %. Chemical formula C_{60}H_{81}NO_{15}S_{2}. $^1$H NMR (400 MHz, CDCl$_3$, ppm): $\delta$ 8.03-8.02 (1H, d), 7.80-7.76 (1H, t), 7.40-7.38 (1H, d), 6.76-6.74 (1H, d), 5.50 (1H, s), 5.29 (1H, s), 4.77-4.76 (1H, m), 4.15-4.12 (2H, m), 4.08 (4H, s), 3.45 (1H, s), 3.00 (1H, m), 2.60-2.58 (1H, t), 2.09 (3H, s), 2.01 (3H, s), 1.98 (3H, s), 0.88-0.83 (13H, m).
Acid 6 was prepared from stearyl alcohol and compound 2 with DMAP as catalyst, in a similar way to the synthesis of compound 3. Next acid 6 was coupled to DOX in the presence of HBTU to obtain the target compound DOX-S-S-SA.

The mixture of stearyl alcohol (0.062 g, 0.23 mmol), compound 2 (prepared from 0.2 g dithiodiglycolic acid [1]) and a catalytic amount of DMAP dissolved in dichloromethane (3 mL) was stirred at room temperature for 5 min. The product (6) was purified by silica gel column chromatography, eluting with a solution of hexane, ethyl acetate and acetic acid. The yield was 49.6 %. $^1$H NMR (400 MHz, CDCl$_3$, ppm): δ 4.17-4.14 (2H, t), 3.63-3.60 (4H, d), 1.67-1.62 (2H, m), 1.26 (35H, br), 0.90-0.86 (3H, t).

DOX (100 mg, 0.18 mmol) was suspended in dry dichloromethane (25 mL). Acid 6 (78.2 mg, 0.18 mmol) and HBTU (230 mg, 0.60 mmol) in dry dichloromethane (20 mL) were added slowly to the reaction mixture. Then, $N,N$-diisopropylethylamine (DIPEA, 200 mg, 1.54 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 3 h under a nitrogen atmosphere. After verification by TLC that the reaction was complete, water (80 mL) was added to the mixture and the crude product was extracted with chloroform (3 x 50 mL). After removal of solvent under reduced pressure, the crude product was purified by silica gel column chromatography, eluting with chloroform/methanol (0-10 % methanol) to give a dark red powder with the yield of 53.4 %. Chemical formula C$_{51}$H$_{73}$N$_{14}$S$_{2}$. $^1$H NMR (400 MHz, CDCl$_3$, ppm):
δ 8.06-8.04 (1H, d), 7.79 (1H, t), 7.40-7.38 (1H, d), 6.89-6.86 (1H, d), 5.53 (1H, m), 5.30 (1H, br),
4.76 (2H, m), 4.54 (1H, s), 4.17-4.15 (5H, m), 4.08 (4H, s), 3.74 (1H, br), 3.57-3.55 (1H, d),
3.40 (2H, m), 3.07 (1H, s), 2.35 (1H, br), 1.55 (2H, br), 1.32-1.25 (44H, m), 0.88 (3H, t).
Psammaplin A (PSA) and psammaplin F (PSF) were synthesized in accordance with a previously reported procedure of ours. [1]
Scheme S7: Synthesis of PSF-D

Scheme S7. Synthetic route to the target compound 3. Reagents and conditions: a) Boc$_2$O, TEA, THF, CH$_3$OH, 0 °C to room temperature, 1 h; b) Cleland’s reagent, 1M KOH, CH$_3$OH, room temperature, 40 min; c) aldrithiol, AcOH, CH$_3$OH, CH$_2$Cl$_2$, N$_2$ atmosphere, room temperature, 24 h; d) CH$_3$OH, room temperature, 10 h.

The following are considerations on the synthesis of compound 3 (PSF-D). It was envisioned that 3 could be synthesized by concise unsymmetrical disulfide coupling$^{[2]}$ between the oximic amide (5) and the amide (8). To start with, compound 1 was reduced by Cleland’s reagent to afford 5 in quantitative yield. Cystamine dihydrochloride (6) was converted to bisamide 7 followed by reduction to yield monoamide 8. 8 was reacted with aldrithiol, obtained from the oxidation of 2-pyridinethiol, to provide pyridyl-disulfide intermediate 9. The convergent synthesis between oximic amide 5 and amide 8 was achieved using simple and mild conditions.

The melting points were determined using an electrically heated X4 digital visual melting point apparatus and were uncorrected. MS data was acquired on a Finnigan MAT mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with initial separation by liquid chromatography (LC–MS). $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker ARX 300 MHz spectrometer (Billerica, MA) at room temperature in DMSO-$d_6$ with tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in ppm (δ). IR spectra (KBr disks) were recorded on a Bruker IFS55 instrument (Billerica, MA). All chemicals were obtained from commercial suppliers and used without purification. TLC analysis was carried out on silica gel plates GF254 (Qingdao Haiyang Chemical Co., Ltd, China). Column chromatography was performed with silica gel 60 (200-300 mesh).

*Synthesis of N,N’-bis(t-butyloxycarbonyl)cystamine (7):* To a solution of cystamine dihydrochloride
(6) (4.00 g, 17.86 mmol) in CH\textsubscript{3}OH (120 mL) was added TEA (11.10 mL, 79.90 mmol), stirring for 5 min. Di-tert-butyl dicarbonate (8.40 g, 29.29 mmol) in THF (24 mL) was then added dropwise, at 0 °C, with stirring. The mixture was then stirred for a further 15 min at 0 °C and at room temperature for 1 h. Upon completion, the solvent was evaporated and the residue redissolved in DCM (120 mL). The organic layer was washed with water (80 mL x 3) and brine (60 mL x 3), and dried over MgSO\textsubscript{4}, resulting in a white solid. Yield was 94 %. mp: 108-109 °C. ESI-MS (m/z): 353.1 [M+H]\textsuperscript{+}. ¹H NMR (300 MHz, CDCl\textsubscript{3}, ppm): δ 1.48 (18H, s, CH\textsubscript{3}×6), 2.80 (4H, t, J = 6.6 Hz, SCH\textsubscript{2}×2), 3.45 (4H, m, CH\textsubscript{2}×2), 5.02 (2H, s, NH×2).

Synthesis of N-t-butyloxycarbonylcysteamine (8): To a solution of 7 (0.32 g, 0.90 mmol) in CH\textsubscript{3}OH (30 mL) were added 1M KOH (90 µL) and Cleland’s reagent (DL-dithiothreitol, 0.42 g, 2.70 mmol) at room temperature. The reaction was quenched with 0.5 M HCl at 0 °C after 1 h and extracted with DCM (30 mL). The organic layer was washed with water (30 mL x 2) and brine (30 mL x 2), and dried over MgSO\textsubscript{4} to produce a yellowish oil. The yield was 99 %. ESI-MS (m/z): 200.0 [M+H]\textsuperscript{+}.

Synthesis of N-t-butyloxycarboryl-2-(2-pyridyldithio)ethanamine (9): To a solution of aldrithiol (1.19 g, 5.40 mmol) in EtOH (15 mL) were added dropwise acetic acid (1.6 mL) and thiol 8 (0.32 g, 1.80 mmol) in EtOH (20 mL) under N\textsubscript{2} atmosphere. The mixture was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was redissolved in DCM (20 mL) and washed with water (20 mL x 3) and brine (20 mL x 3), and then dried over MgSO\textsubscript{4}, filtered and evaporated. The residue was purified by silica gel column chromatographic using petroleum ether and acetone (16:1, v/v) as eluent to give a yellowish oil. The yield was 86 %. ESI-MS (m/z): 287.1 [M+H]\textsuperscript{+}. ¹H NMR (300 MHz, DMSO-d\textsubscript{6}, ppm): δ 1.35 (9H, s, CH\textsubscript{3}×6), 2.86 (2H, t, J = 6.8 Hz, SCH\textsubscript{2}), 3.19 (2H, m, CH\textsubscript{2}), 7.02-7.81 (4H, m, Py-H), 8.44 (1H, t, J = 6.0 Hz, NH).

Synthesis of (E)-N-3-(3-bromo-4-hydroxyphenyl)-2-hydroxyiminocysteamine (5): To a solution of psammaplin A (PSA) (1) (0.60 g, 0.90 mmol) in CH\textsubscript{3}OH (30 mL) were added 1M KOH (90 µL) and Cleland’s reagent (0.42 g, 2.70 mmol) at room temperature. The reaction was quenched with 0.5 M HCl at 0 °C after 1 h and extracted with DCM (30 mL). The organic layer was washed with water (30 mL x 2) and brine (30 mL x 2), and then dried over MgSO\textsubscript{4}, filtered and concentrated. The
desired compound 5, obtained as a yellow oil (0.60 g), was used directly in the next reaction. The yield was 99 %. ESI-MS (m/z): 333.0 [M+H]⁺. IR (KBr, cm⁻¹): 3384, 2930, 1655, 1527, 1493, 1421, 1363, 1285, 1214, 1043, 1009, 801. ¹H NMR (600 MHz, DMSO-d₆, ppm) δ: 2.24 (1H, t, J = 7.5 Hz, SH), 2.54 (2H, q, J = 7.5 Hz, CH₂), 3.28 (2H, m, CH₂), 3.69 (2H, s, CH₂), 6.83 (1H, d, J = 8.4 Hz, Ph-5’), 7.00 (1H, dd, J = 8.4 Hz, 2.1 Hz, Ph-6’), 7.29 (1H, d, J = 2.1 Hz, Ph-2’), 8.05 (1H, t, J = 6.0 Hz, NH), 9.99 (1H, s, Ph-OH), 11.8 (1H, s, NOH). ¹³C NMR (150 MHz, DMSO-d₆, ppm) δ: 27.3, 28.0, 42.4, 109.2, 116.5, 129.1, 129.5, 133.1, 152.2, 152.7, 163.5.  

**Synthesis of** ((E)-N-(3-bromo-4-hydroxyphenyl)-2-oximidopropionyl-N’-t-butyloxycarboryl)cystamine (3): To a solution of pyridyl-disulfide 9 (0.26 g, 0.90 mmol) in EtOH (20 mL) was added dropwise a solution of oximic amide 5 (0.10 g, 0.30 mmol) in EtOH (10 mL) under an N₂ atmosphere. The mixture was stirred for 12 h at room temperature. After removal of the solvent, the residue was redisolved in DCM (30 mL), washed with water (30 mL x 2) and brine (30 mL x 2), and dried over MgSO₄. The organic layer was concentrated and the crude product purified by silica gel column chromatography using petroleum ether and acetone (3:1, v/v) as eluent to give a white solid. The yield was 60 %. Chemical formula C₁₈H₂₆BrN₃O₅S₂. mp: 73-74 °C. ESI-MS (m/z): 505.9 [M-H]. IR (KBr, cm⁻¹): 3316, 2974, 2929, 1704, 1665, 1528, 1421, 1366, 1283, 1253, 1221, 1166, 1045, 1017, 985. ¹H NMR (300 MHz, DMSO-d₆, ppm) δ: 1.36 (9H, s, CH₃×3), 2.73 (2H, t, J = 6.8 Hz, SCH₂), 2.79 (2H, t, J = 6.8 Hz, CH₂), 3.18 (2H, m, CH₂), 3.38 (2H, m, CH₂), 3.67 (2H, s, PhCH₂), 6.81 (1H, d, J = 8.1 Hz, Ph-5’), 6.95 (1H, t, J = 5.4 Hz, NH), 7.00 (1H, dd, J = 8.1 Hz, 1.8 Hz, Ph-6’), 7.27 (1H, d, J = 1.8 Hz, Ph-2’), 8.06 (1H, t, J = 5.7 Hz, NH), 10.0 (1H, s, Ph-OH), 11.8 (1H, s, NOH).
Scheme S8: Synthesis of PSA-D

Scheme S8. Synthetic route to the target compound 4 (PSA-D). Reagents and conditions: a) K$_2$CO$_3$, TEBA, Br(CH$_2$)$_6$Br, 100 °C, 3 h; b) 2 M NaOH aq., CH$_3$OH, room temperature, 4 h; c) phenylamine, HOBT, EDCI, THF, 0 °C to room temperature, 24 h; d) KSAc, C$_2$H$_5$OH, 60 °C, 4 h; e) 1.2 M LiOH aq., C$_2$H$_5$OH, N$_2$ atmosphere, room temperature, 4 h; f) aldrithiol, acetic acid, CH$_3$OH, CH$_2$Cl$_2$, N$_2$ atmosphere, room temperature, 3 h; g) CH$_3$OH, CH$_2$Cl$_2$, room temperature, 8 h.

The following is considerations on the synthesis of compound 4 (PSA-D). Taking advantage of the convergent synthesis method, diethyl malonate (10) was alkylated, hydrolyzed, amidated, nucleophilic substituted and hydrolyzed, to form compound 15. This was reacted with aldrithiol, obtained from the oxidation of 2-pyridinethiol, to provide pyridyl-disulfide intermediate 16. The final disulfide 4 was obtained by disulfide coupling between the oximic amide 5 and the pyridyl-disulfide 16 in simple and mild conditions with good yields.

**Synthesis of diethyl 2-(6-bromohexyl)malonate (11):** A mixture of 1,6-dibromohexane (5.00 g, 20.49 mmol), TEBA (0.025 g, 0.11 mmol) and K$_2$CO$_3$ (8.49 g, 61.47 mmol) was heated at 100 °C. Diethyl malonate (10) (9.84 g, 61.47 mmol) was then added dropwise. The mixture was stirred for 2 h at 100 °C, then filtered and evaporated at 140 °C. The residue was purified by silica gel column chromatography using petroleum ether as eluent to give a colorless oil. The yield was 74 %. ESI-MS (m/z): 323.1 [M+H]$^+$.

**Synthesis of 2-(6-bromohexyl)malonic acid (12):** To a solution of diethyl maleate 11 (2.3 g, 7.1 mmol) in CH$_3$OH (40 mL) was added dropwise a solution of NaOH (1.10 g, 28.4 mmol) in CH$_3$OH (14 mL). The mixture was stirred for 3 h at room temperature. After removal of the solvent,
the residue was redissolved in H$_2$O (100 mL) and washed with Et$_2$O (60 mL x 3). The aqueous layer was adjusted to pH = 2 with 0.5 M HCl, extracted with ethyl acetate (80 mL x 3) and dried over MgSO$_4$. The organic layer was concentrated to afford the crude product as a white solid. The yield was 82 %. ESI-MS (m/z): 265.0 [M-H]$^-$.

**Synthesis of 2-(6-bromohexyl)-N,N’-diphenylmalonamide (13):** To a solution of malonic acid 12 (0.9 g, 3.4 mmol) in THF (70 mL) was added a HOBT (1.00 g, 7.5 mmol) at room temperature, after stirring for 40 min, phenylamine (0.7 g, 7.5 mmol) was added dropwise at 0 °C, followed by addition of EDCI (1.4 g, 7.5 mmol). The resulting mixture was stirred for 1 h and the temperature was then allowed to warm to room temperature for 24 h. After removal of the solvent, the residue was redissolved in ethyl acetate (70 mL) and washed with brine (50 mL x 3), dried over MgSO$_4$, and filtered and concentrated. The residue was purified by silica gel column chromatography using petroleum ether and acetone (9:1, v/v) as eluent to give a white solid. The yield was 62 %. ESI-MS (m/z): 419.1 [M+H]$^+$. 

**Synthesis of S-6,6-bis(phenylcarbamoyl)hexylthioethyl (14):** A solution of diphenylmalonamide 13 (0.82 g, 2.20 mmol) in EtOH (50 mL) was heated to 60 °C. Potassium thioacetate (0.40 g, 3.52 mmol) was added, and the mixture was stirred for 4 h. After removal of the solvent, the residue was redissolved in DCM (80 mL) and washed with water (100 mL x 3) and brine (80 mL x 3), and dried over MgSO$_4$. This was followed by filtration and concentration in a vacuum. The residue was purified by silica gel column chromatography using petroleum ether and acetone (6:1, v/v) as eluent to give a white solid. The yield was 80 %. mp: 122-123 °C. IR (KBr, cm$^{-1}$): 3276, 2926, 2854, 1677, 1600, 1536, 1499, 1443, 1352, 1249, 1136, 753, 692. ESI-MS (m/z): 413.2 [M+H]$^+$. $^1$H NMR (300 MHz, CDCl$_3$, ppm) $\delta$: 1.28-1.48 (8H, m, CH$_2$×4), 2.07 (2H, q, $J$ = 7.2 Hz, CH$_2$), 2.31 (3H, s, CH$_3$), 2.79 (2H, t, $J$ = 6.7 Hz, CH$_2$), 3.51 (1H, s, CH), 7.16 (2H, t, $J$ = 7.4 Hz, Ph-4’×2), 7.36 (4H, t, $J$ = 7.7 Hz, Ph-3’,5’×2), 7.63 (4H, d, $J$ = 8.0 Hz, Ph-2’,6’×2), 9.47 (2H, s, 2NH).

**Synthesis of S-6,6-bis(phenylcarbamoyl)hexylthiol (15):** To a solution of thioester 14 (0.40 g, 1.00 mmol) in EtOH (35 mL) was added dropwise a solution of LiOH·H$_2$O (8 mL, 1.20 M) under an N$_2$ atmosphere. The mixture was stirred for 5 h at room temperature. The reaction was adjusted to pH = 1 with 0.5 M HCl at 0 °C and extracted with DCM (60 mL x 3). The organic layer was
washed with water (60 mL x 3), dried over MgSO₄, filtered and concentrated. The residue was purified by silica gel column chromatographic using petroleum ether and acetone (8:1, v/v) as eluent to give a white solid. Yield was 87 %. mp: 153-154 °C. IR (KBr, cm⁻¹): 3271, 3058, 2921, 2852, 1674, 1599, 1533, 1499, 1444, 1353, 1252, 1167, 976, 752, 693. ESI-MS (m/z): 371.2 [M+H]⁺. ¹H NMR (300 MHz, DMSO-d₆) δ: 1.31 (6H, m, CH₂×3), 1.49 (2H, m, CH₂), 1.89 (2H, m, CH₂), 2.18 (1H, t, J = 7.8 Hz, SH), 2.43 (2H, t, J = 7.2 Hz, SCH₂), 3.47 (1H, t, J = 7.1 Hz, CH), 7.04 (2H, t, J = 7.2 Hz, Ph-4’×2), 7.30 (4H, t, J = 7.8 Hz, Ph-3’, 5’×2), 7.60 (4H, d, J = 7.8 Hz, 2Ph-2’, 6’×2), 9.93 (2H, s, NH×2). ¹³C NMR (75 MHz, DMSO-d₆) δ: 24.0, 27.4, 27.9, 28.7, 29.9, 33.6, 55.4, 119.6, 123.8, 129.0, 139.2, 168.2.

**Synthesis of N,N’-diphenyl-2-(6-(pyridin-2-yldisulfanyl)hexyl)malonamide (16):** Compound 16 was synthesized using similar procedures to those employed for compound 9, using compound 15 as starting material, to produce a white solid. The yield was 85 %. mp: 48-49 °C, IR (KBr, cm⁻¹): 3279, 3137, 3043, 2926, 2855, 1676, 1601, 1543, 1498, 1443, 1416, 1310, 1250, 1174, 1178, 755. ESI-MS m/z: 466.2 [M+H]⁺. ¹H NMR (300 MHz, DMSO-d₆, ppm) δ: 1.27 (4H, m, CH₂×2), 1.62 (2H, t, J = 6.3 Hz, CH₂), 1.91 (2H, m, CH₂), 2.81 (2H, t, J = 6.9 Hz, SCH₂), 3.49 (1H, t, J = 6.9 Hz, CH), 7.05 (2H, t, J = 7.2 Hz, Ph-4’×2), 7.16 (1H, t, J = 4.8 Hz, Py-5’), 7.30 (4H, t, J = 7.8 Hz, Ph-3’, 5’×2), 7.63 (4H, d, J = 7.8 Hz, Ph-2’, 6’×2), 7.76 (2H, m, Py-3’, 4’), 8.41 (1H, d, J = 4.5 Hz, Py-6’), 9.96 (2H, s, NH×2).

**Synthesis of (E)-2-(6-((2-(3-(3-bromo-4-hydroxyphenyl)-2-(hydroxymino)propanamido)ethyl)disulfanyl)hexyl)-N,N’-diphenylmalonamide (4):** Compound 4 was synthesized by similar procedures to those employed for compound 3, using compound 16 as starting material, to give a white solid. Yield was 58 %. Chemical formula C₃₂H₃₇BrN₄O₅S₂. mp: 47-48 °C. IR (KBr, cm⁻¹): 3398, 2925, 2853, 1666, 1600, 1538, 1443, 1118, 755. ESI-MS m/z: 699.7 [M-H]⁻. ¹H NMR (300 MHz, DMSO-d₆, ppm) δ: 1.31 (6H, m, CH₂×3), 1.56 (2H, m, CH₂), 1.89 (2H, m, CH₂), 2.67 (2H, t, J = 7.1 Hz, CH₂), 2.77 (2H, t, J = 6.9 Hz, SCH₂), 3.42 (2H, m, CH₂), 3.47 (1H, t, J = 7.5 Hz, CH), 3.67 (2H, s, PhCH₂), 6.81-7.59 (13H, m, Ph-H×13), 8.06 (1H, t, J = 5.7 Hz, CONH), 9.92 (2H, s, PhNH×2), 10.0 (1H, s, PhOH), 11.8 (1H, s, NOH).
Scheme S9. Synthetic route to the target compound SRB-S-S-VE. Reagents and conditions: (a) CH$_2$Cl$_2$, oxalyl chloride, DMF, room temperature, 16 h; (b) CH$_2$Cl$_2$, DMAP, N-Boc-ethylenediamine, triethylamine, room temperature, 18 h; (c) CH$_2$Cl$_2$, trifluoroacetic acid, 0 °C, 2 h; (d) VE-CO-CH$_2$-S-S-CH$_2$-COOH, HBTU, DIPEA, room temperature, 4 h.

To a stirred solution of sulforhodamine B sodium salt (580 mg, 1 mmol) in CH$_2$Cl$_2$ (25 mL) at 0 °C, was added, sequentially, oxalyl chloride (430 µL, 5 mmol) slowly and a catalytic amount of anhydrous DMF (12 µL). The resulting mixture was stirred at room temperature for 16 h, and then the reaction was concentrated in vacuo and dried under vacuum to yield 17. Yield was 98 %.

N-Boc-ethylenediamine (160 mg, 1 mmol), DMAP (6.1 mg, 0.05 mmol) and triethylamine
(420 µl, 3 mmol) were dissolved in CH₂Cl₂ (20 mL) and then was added dropwise into a stirred solution of the above sulfonyl chloride 17 in CH₂Cl₂ (50 mL) at 0 °C. The reaction was allowed to warm to room temperature and then, after 13 h, the reaction was washed with 5 % HCl (50 mL), water (50 mL) and dried over MgSO₄. The organic solution was concentrated and purified by silica gel column chromatography using CHCl₃:CH₃OH (100:3, v/v) as eluent to give a purple solid 18. Yield was 46 %.

The Boc-protected amine 18 (300 mg, 0.43 mmol) was dissolved in CH₂Cl₂ (35 mL) and then treated with trifluoroacetic acid (3 mL) at 0 °C for 2 h. The solvent and trifluoroacetic acid were removed by concentration under vacuum. The residue was dissolved in CH₂Cl₂ (80 mL) and washed with saturated sodium carbonate solution (25 mL). The organic phase was dried over MgSO₄ and concentrated to obtain the amine 19. Yield was 65 %.

The compound 19 (33 mg, 0.055 mmol), VEfCOH₂-S-SfCH₂fCOOH (compound 3, 30 mg, 0.05 mmol) and HBTU (38 mg, 0.1 mmol) were dissolved in CH₂Cl₂ (25 mL) at 0 °C and stirred under N₂. After 0.5 h, DIPEA (17.5 µL, 0.1 mmol) was added dropwise. The solution was allowed to react at room temperature under N₂ for 5 h, and washed with 5 % HCl (10 mL), water (10 mL) and dried over MgSO₄. The solvent was removed under vacuum and the product was separated by chromatography on silica using 3 % MeOH in CHCl₃ as eluent. Yield: 62 %, purple solid. Chemical formula C₆₂H₈₈N₄O₁₀S₄. MS (m/z): 1199.21 [M+Na]+. ¹H NMR (400 MHz, CDCl₃, ppm): δ 0.80-0.84 (12H, m), 1.03-1.27 (33H, m), 1.49 (3H, m), 1.72 (2H, m), 1.91-2.04 (9H, m), 2.52 (2H, m), 2.77 (2H, s), 3.13 (2H, s), 3.24 (2H, s), 3.50-3.58 (8H, m), 3.80 (2H, s), 6.63 (2H, d), 6.98 (2H, d), 7.18 (1H, d), 7.43 (2H, d), 7.88 (1H, d), 8.65 (1H, s).
SRB-VE was synthesized in accordance with a previously reported procedure of ours.⁵
Scheme S10: Synthesis of 5-FU-S-S-SA

Scheme S10. Synthetic route to the target compound 5-FU-S-S-SA. Reagents and conditions: a) CH$_3$CN, CH$_2$Cl$_2$, DCC, DMAP, room temperature, 24 h.

In the presence of DCC and DMAP, the 1 position of 1,3-dihydroxymethyl-5-fluorouracil was mainly substituted when compound 6 was added.

**Synthesis of 5-FU-S-S-SA:** 1,3-Dihydroxymethyl-5-fluorouracil (19 mg, 0.1 mmol) was dissolved in a mixture of CH$_2$Cl$_2$ and CH$_3$CN (5:1, v/v) (12 mL), and o-octadecyl dithiodiglycolic acid (6) (52 mg, 0.12 mmol) and DCC (62 mg, 0.3 mmol) were added. After the compounds had dissolved, a catalytic amount of DMAP was added. The solution was stirred at room temperature for 24 h. The solid was filtered and the filtrate was concentrated and purified by silica gel column chromatography using CHCl$_3$ and CH$_3$OH (100:1, v/v) as eluent to give a white solid. Yield was 30%. Chemical formula C$_{28}$H$_{47}$FN$_2$O$_7$S$_2$. MS (m/z): 629.21 [M+Na]$^+$. $^1$H NMR (400 MHz, CDCl$_3$, ppm): δ 0.84 (m, CH$_3$), 1.22 (m, (CH$_2$)$_{16}$), 1.64 (m, CH$_2$), 1.90 (m, CH$_2$), 3.56 (m, CH$_2$), 4.10 (m, CH$_2$), 5.67(s, CH$_2$), 7.61 (d, =CH-).
Scheme S11: Synthesis of 5-FU-SA

Scheme S11. Synthetic route to the target compound 5-FU-SA. Reagents and conditions: a) CH₂Cl₂, DCC, DMAP, room temperature, 24 h.

Synthesis of 5-FU-SA: 1,3-Dihydroxymethyl-5-fluorouracil was reacted with stearic acid, in the presence of DCC and DMAP, to afford the ester. Stearic acid (284 mg, 1 mmol) and DCC (206 mg, 1 mmol) were added to a solution of 1,3-dihydroxymethyl-5-fluorouracil (210 mg, 1.1 mmol) in a solvent mix of CH₂Cl₂ and CH₃CN (5:1, v/v) (24 mL). A catalytic amount of DMAP was then added. After the mixture was stirred at room temperature for 24 h, the solution was filtered and the filtration was washed with 5 % NaHCO₃ (20 mL) and water (20 mL), and then dried over MgSO₄. The organic solution was concentrated and purified by silica gel column chromatography using CHCl₃ and CH₃OH (100:1, v/v) as eluent to give a white powder. Yield was 60 %. Chemical formula C₂₄H₄₁FN₂O₅. MS (m/z): 479.32 [M+Na]⁺. ¹H NMR (400 MHz, CDCl₃, ppm): δ 0.84 (3H, m, CH₃), 1.22 (30H, m, (CH₂)₁₅), 1.62 (2H, m, CH₂), 2.35 (2H, m, CH₂), 5.60 (2H, s, CH₂), 7.58 (1H, d, =CH-).
Scheme S12: Synthesis of GEM-S-S-SA

Scheme S12. Synthetic route to the target compound GEM-S-S-SA. Reagents and conditions: a) DMF, HBTU, DIPEA, room temperature, 18 h.

**Synthesis of GEM-S-S-SA:** Gemcitabine (GEM) (79 mg, 0.3 mmol) was dissolved in 5 mL of anhydrous DMF. O-octadecyl dithiodiglycolic acid (6) (43.4 mg, 0.1 mmol) and HBTU (76 mg, 0.2 mmol) were dissolved in 5 mL anhydrous DMF and added to the GEM solution by syringe. Diisopropyl ethyl amine (35 µL, 0.2 mmol) was added dropwise under ice bath and N₂. The solution was stirred under N₂ at room temperature for 18 h, then diluted with 50 mL EtOAc, washed with 5 % HCl (20 mL), 5 % NaHCO₃ (20 mL), and brine (20 mL), and then dried over MgSO₄. The solvent was removed under vacuum and the product was separated by chromatography on silica with 10 % MeOH in CHCl₃ as eluent. A white solid resulted with yield of 51 %. Chemical formula C₃₁H₅₁F₂N₃O₇S₂. MS (m/z): 702.12 [M+Na]⁺. ¹H NMR (400 MHz, DMSO-d₆, ppm): δ 0.80 (3H, m, CH₃), 1.18 (32H, m, (CH₂)₁₆), 1.50 (2H, m, CH₂), 3.56-4.20 (8H, m, 3’-H, 4’-H, 5’-CH₂-,CH₂-S-S-CH₂), 5.22 (1H, s, 5’-OH), 6.12 (1H, m, 1’-H), 6.24 (1H, s, 3’-OH), 7.17 (1H, d, =CH-), 8.23 (1H, d, =CH-), 11.16 (1H, s, -NH-C=O).
Supporting Figure S1

Figure S1. Comparison of hydrophobicity of PTX-VE and PTX-S-S-VE using HPLC.

Retention times were 32.37 and 33.32 min for PTX-VE and PTX-S-S-VE, respectively. The HPLC system consisted of a Waters 2487 Dual λ Absorption Detector and a Waters 600 pump (Waters Corporation, Milford, MA). The separation was carried out on an ODS HYPERSIL (3 µm particle size, 2.1 mm × 100 mm) column (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 0.5 mL/min. Compound concentration was determined at a UV wavelength of 228 nm. Injection volume was 10 µL. Gradient elution was applied with a mobile phase of ACN and water. Initial mobile phase composition was 40 % ACN from 0 to 36 min, to 100 % ACN at 37 min for 30 min, and back to the initial conditions at 67 min. Equilibration time was 9 min giving a total run time of 76 min.
Supporting Figure S2

**PTX-S-S-VE DSINMs**

- Z-Average (d, nm): 116.5
- PdI: 0.130
- Intercept: 0.896
- Result quality: Good

**DOX-S-S-SA DSINMs**

- Z-Average (d, nm): 148.1
- PdI: 0.065
- Intercept: 0.943
- Result quality: Good

**5-FU-S-S-SA DSINMs**

- Z-Average (d, nm): 111.9
- PdI: 0.117
- Intercept: 0.935
- Result quality: Good
Figure S2. Particle size distributions of PTX-S-VE, DOX-S-SA, 5-FU-S-SA, PSF-D, DOX-S-S-VE, PSA, PSA-D and PEGylated PTX-S-S-VE DSINMs were determined by dynamic light scattering (DLS) analysis using a Submicron Particle Sizer (NICOMP, Autodilute-PAT model 370, Particle Sizing Systems, Port Richey, FL) operated in the NICOMP mode.
Supporting Figure S3

DSINMs for combined drug delivery (DOX and 5-FU)

When nanocarriers are designed to deliver combined drugs to target cancer cells, the drugs will ideally support synergistic effects within the signaling pathways of the cells. The development of nanocarriers, however, that exhibit a high loading efficiency for a combination of a hydrophobic drug and a hydrophilic drug poses challenges. Thus, we explored the potential to combine 5-FU and DOX in a single, stable nanoparticle. After conjugating a water soluble drug, 5-FU, to SA through an S-S bond to form a hydrophobic prodrug (5-FU-S-S-SA, Scheme S10), homogeneous DSINMs were self-assembled with cubic-shaped morphology, a size of 111.9 nm (Figure S2 and S3A), and a Zeta potential of 31 mV. In contrast, the non S-S bonded prodrug, 5-FU-SA (Scheme S11), formed aggregates (Figure S3A). DSINMs for combined delivery of DOX-S-S-SA with 5-FU-S-S-SA (DOX/5-FU DSINMs, 1/1 mol/mol), were confirmed by TEM imaging to have spherical morphology (Figure S3B). As expected, the physical mixing of DSINMs (DOX and 5-FU DSINMs prepared separately, then mixed), resulted in DSINMs that exhibited both cubic and spherical morphologies (data now shown), conveying the stability of each particle post-formulation. The DSINMs carrying combined drugs were further investigated by sucrose density gradient centrifugation (SDGC), which successfully separated DOX, 5-FU and DOX/5-FU DSINMs (Figure S3, C and D). 5-FU DSINMs (alone and mixed with DOX DSINMs) were suspended in the 0 % glucose fraction, observed via the Tyndall effect (Figure S3C). DOX DSINMs, however, were visualized as a red fluorescent pellet at the bottom of the tubes for DOX DSINMs alone and for the mixture (Figure S3D). Both the Tyndall effect in the 0 % fraction and the red pellet disappeared for DOX/5-FU DSINMs which were detected in the layer between the 40 % and 60 % glucose fractions (Figure S3E). DSINMs co-carrying PTX-S-S-VE and DOX S-S-VE (Figure S4) or GEM-S-S-SA (Scheme S12) were also obtained. All of this data confirmed that DSINMs are capable of delivering combined drugs.
Figure S3 DSINMs for combined drug delivery.

A, Molecular structure of 5-FU-S-S-SA and 5-FU-SA and corresponding TEM images after dispersion in water. B, TEM images of DOX/5-FU DSINMs (1/1 mol/mol). C-E, Separation of DSINMs by sucrose density gradient centrifugation (SDGC). C, Photographs of Tyndall scattering were taken from the 0 % glucose fraction after SDGC with samples of DOX/5-FU DSINMs (1), DOX DSINMs (2), the physical mixture of DOX DSINMs and 5-FU DSINMs (3) and 5-FU DSINMs (4). D, E: Photo imaging of the bottom and body of ultracentrifuge tubes representing DOX DSINMs (1), 5-FU DSINMs (2), DOX/5-FU DSINMs (3) and the physical mixture of DOX DSINMs and 5-FU DSINMs (4).
Supporting Figure S4

DSINMs for combined drug delivery (DOX and PTX)
DSINMs co-delivering prodrugs of PTX and DOX have been explored. As shown in Figure S4, homogeneous DSINMs that contain PTX-S-S-VE and DOX-S-S-VE (1/1, mol/mol) were obtained, with a particle size of 90 nm. To confirm that both drugs were co-delivered by the DSINMs, instead of the physical mixing of PTX-DSINMs and DOX-DSINMs, SDGC (sucrose density gradient centrifugation) was employed. PTX-DSINMs were detected in the 50% glucose fraction by the Tyndall effect (Figure S4C, number 1), and DOX-DSINMs were observed at the bottom of the ultracentrifuge tube (Figure S4D, number 2). However, the Tyndall effect disappeared in the sample of the DSINMs containing both drugs (Figure S4C, number 3) which were detected at the bottom of the ultracentrifuge tube (Figure S4D, number 3), with > 96% recovery for both drugs. All of this data indicates that the combined delivery of PTX and DOX can be achieved by DSINMs.
Figure S5. Biodistribution profiles of DOX (1) and DOX-S-S-SA DSINMs with (2) and without (3) PEGylation. a, biodistribution imaging; b, calculated blood concentration of the tested samples. Mouse organs and blood were collected 3 h post i.v. injection. DOX dose was 2.5 mg/kg. PEGylated DOX-S-S-SA DSINMs dose was 4.14 mg/kg, equivalent to 2.5 mg/kg of DOX.
Supporting Figure S6

Figure S6. Body weight of tumor bearing mice after IV drug administration.
Supporting Figure S7

Figure S7. In vitro evaluation of drug-induced hemolysis.

Drug induced hemolysis in vitro is a simple and reliable estimation of the membrane damage caused by the drug or its formulation in vivo. The hemolytic activity of the PTX-S-S-VE DSINMs and Taxol in mouse erythrocyte suspension is shown in Supporting Figure S7. PTX-S-S-VE DSINMs showed significantly less hemolysis than Taxol at a concentration of 0.4 mM (p < 0.01, Student’s t-test, paired, two sided). On increasing the concentration of each to 0.8 mM (equivalent to an IV dose of 50 mg/kg PTX), ~100 % hemolysis was observed with Taxol but only 20 % hemolysis was observed with PTX-S-S-VE DSINMs.
Figure S8. TEM image of SRB-VE (A) and SRB-S-S-VE DSINMs (B).
Supporting Figure S9

Figure S9. Tumor imaging in live mice. The tumor (indicated by arrows) bearing mice were imaged 8, 12 and 48 h after injection of free SRB and PEGylated SRB-S-S-VE DSINMs. The images at 2 and 4 h are shown in Figure 5 (main text).
**Supporting Table S1**
Inhibitory activities of small molecular compounds containing disulfide bond against HDACs and the proliferation of MCF-7 and HL-60.

| Compounds | HDACs inhibition $^{a,b}$ (%) | MCF-7 IC$_{50}$$^b$ (µM) | HL-60 GI$_{50}$$^b$ (µM) |
|-----------|-------------------------------|---------------------------|---------------------------|
| PSF       | 40.75±3.22                    | 2.26±0.26                 | 0.19±0.02                 |
| PSA       | 21.33±1.86                    | 20.73±3.23                | 2.43±0.17                 |
| PSF-D     | 52.78±5.34                    | 11.29±0.49                | 0.38±0.01                 |
| PSA-D     | 72.63±3.57                    | 2.52±0.13                 | 0.25±0.02                 |

$^a$ HDACs inhibition: the inhibition rate of the compounds against HDACs at a concentration of 30 nM.

$^b$ Data shown are means ± SD of three independent experiments.
Supporting Table S2

**PK parameters of PTX and prodrug in CD-1 mice after IV injection administration.**

| Parameters | Unit | Pegylated PTX-S-S-VE DSINMs | Taxol | P-Value |
|------------|------|-----------------------------|-------|---------|
| AUC(0-t)   | mg/L*h | 328.81±39.91 | 1.31±0.33 | 0.0049 |
| MRT(0-t)   | h    | 10.43±0.33    | 1.20±0.06 | 0.0003 |
| $t_{1/2}$  | h    | 25.74±7.66    | 1.47±0.16 | 0.0328 |
| Vz         | L/kg | 0.45±0.09     | 8.01±1.21 | 0.0075 |
| CLz        | L/h/kg | 0.01±0.00  | 3.85±0.97 | 0.0208 |

Parameters: AUC, area under the curve; MRT, mean residence time; $t_{1/2}$, half-life; Vz, volume of distribution; CLz, total body clearance.
$^1$H NMR spectra

PTX-S-S-VE

400 MHz, CDCl$_3$, 25 °C, 10 mg/mL
DOX-S-S-VE

400 MHz, CDCl₃, 25 °C , 10 mg/mL
DOX-S-S-SA

400 MHz, CDCl₃, 25 °C, 10 mg/mL
PSA

300 MHz, CD$_3$OD, 25 °C, 5 mg/mL
PSF

600 MHz, CD$_3$OD, 25 °C , 5 mg/mL
PSA-D

300 MHz, DMSO-d$_6$, 25 °C, 5 mg/mL.
PSF-D

300 MHz, DMSO-$d_6$, 25 °C, 5 mg/mL
5-FU-SA

400 MHz, CDCl₃, 25 °C, 5 mg/mL
5-FU-S-S-SA

400 MHz, CDCl₃, 25 °C, 5 mg/mL
GEM-S-S-SA

400 MHz, DMSO-$d_6$, 25 °C, 5 mg/mL
SRB-S-S-VE

400 MHz, CDCl₃, 25 °C, 10 mg/mL
Supporting References

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