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Cancer Cell Surface Induced Peptide Folding Allows Intracellular Translocation of Drug

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Abstract

Many lead molecules identified in drug discovery campaigns are eliminated from consideration due to poor solubility and low cell permeability. These orphaned molecules could have clinical value if solubilized and delivered properly. SVS-1 is a de novo designed peptide that preferentially folds at the surface of tumor cells, adopting a β -hairpin conformation that rapidly translocates into the cytoplasm, and ultimately nucleus, of cells. SVS-1 is stable in serum and small molecules attached to the peptide are effectively delivered to cancer cells via mechanisms involving physical translocation and, to a lesser extent, clathrin-dependent endocytosis. For example, ligating the model hydrophobic drug Paclitaxel (PTX) to SVS-1 improved its aqueous solubility by ~1000-fold and successfully delivered and released PTX to cancer cells in vitro and tumors in vivo without toxic adjuvants. These results suggest SVS-1 can serve as a simple, effective delivery platform for molecules with poor solubility and permeability.

Graphical abstract

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Keywords

Cell-penetrating peptide; Translocation; Biodistribution; Drug Delivery; Paclitaxel; In vivo Efficacy

1. Introduction

Developing methods to deliver bioactive small molecules preferentially to target cells remains a daunting challenge despite recent advances in drug delivery. Furthermore, many potential lead molecules identified in high-throughput drug discovery campaigns are dropped early from consideration due to issues of poor water solubility and/or low cellular permeability. This class of orphaned molecules could in fact have potent in vivo activities if solubilized and delivered properly. The development of new delivery vehicles that are selective in their action begin to address this need.

Our lab recently reported the *de novo* design of an anticancer peptide, named SVS-1. This peptide kills cancer cells via a lytic mechanism that involves its cell surface-induced folding [1]. Herein, we report that when cancer cells are presented with concentrations of SVS-1 that are below its IC₅₀ for lytic action, the peptide does not kill the cells but rather rapidly translocates across the cell membrane into the cytoplasm, and ultimately the nucleus. We show that cell-surface binding triggers the folding of the peptide into a β -hairpin conformation that rapidly partitions into the membrane. Thus, the cell-surface β -folding event triggers the internalization activity of SVS-1, a mechanism unique to this peptide. When a drug is attached to SVS-1, this serves as an effective means for its intracellular delivery (Fig. 1).

SVS-1 (KVKVKVKV^DPPTKVKVKV-NH₂) is an 18 amino acid peptide designed to bind to, and fold at, negatively charged cancer cell surfaces [1, 2]. The peptide contains two strands of alternating lysine and valine residues, which flank a tetrapeptide motif (-V^DPPT-) designed to adopt a type II' β -turn when the peptide is folded. Previous studies have shown that in the absence of a cell surface, the SVS-1 peptide adopts an ensemble of random coil, bio-inactive conformations [1]. Electrostatic repulsion between the peptide's charged lysine side chains keeps it in the unfolded state. However, when presented with a negatively charged surface, such as that displayed by malignant cells, these side chains electrostatically engage the anionic lipid head groups and glycans at the cell's surface. This binding event triggers the folding of the β -hairpin, where the lysine and valine residues are displayed from opposite faces of the folded conformer. In this folding mechanism, the lysine-rich face of the hairpin is engaged in electrostatic interactions with the cell and its valine face is solvated by water. Solvation of the valine-rich hydrophobic face is entropically unfavorable and, as a result, the folded peptide interpolates into the membrane to release the ordered water. We discovered that at peptide concentrations below that needed to induce SVS-1's lytic action, the peptide rapidly enters cells without effecting cell viability. Studies using differentially labeled analogs and enantiomeric peptides show that SVS-1 rapidly and preferentially penetrates cancer cells through mechanisms involving physical translocation and to a lesser extent clathrin-dependent endocytosis. Ligating the model hydrophobic drug Paclitaxel

(PTX) to SVS-1 improved its aqueous solubility by ~1000-fold and successfully delivered and released PTX to cancer cells *in vitro*. Although SVS-1 is moderately stable to serum proteolysis ($t_{1/2} \sim 6\text{h}$), its *D*-enantiomer displays a half-life well over 8 hours. Importantly, the high solubility of the peptide allows PTX to be administered *in vivo*, without the need for toxic adjuvants, successfully reducing tumor burden in a xenograft mouse model. These results suggest SVS-1 can serve as a simple, effective delivery platform for molecules with poor solubility and permeability.

2. Experimental

2.1 Materials

Fmoc-protected amino acids were purchased from Novabiochem. PL-Rink resin was purchased from Polymer Laboratories. 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) was obtained from Peptides International, and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) was purchased from Chem Impex International. Trifluoroacetic acid was obtained from Acros organics. 1,2-ethanedithiol was purchased from Fluka. Fluorescein-NHS was obtained from Pierce, and Cy5-NHS was purchased from Lumiprobe. Lab-Tek™ 4-well chambered #1 borosilicate glass slides, 24 well cell culture plates, diethyl ether, N-Methylpyrrolidone (NMP) and potassium fluoride (KF) were purchased from Fisher Scientific. 2-mercaptophenylacetic acid was purchased from Santa Cruz Biotechnology. Thioanisole, anisole, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 3-(tritylthio)propionic acid, dimethyl sulfoxide (DMSO), 4-dimethylaminopyridine (DMAP), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 200 mM glutamine solution were obtained from Sigma-Aldrich. RPMI-1640 media and Hoechst 33342 trihydrochloride dye was purchased from Invitrogen. Heat inactivated fetal bovine serum (FBS) and trypsin EDTA were obtained from Hyclone Laboratory Inc. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids. Pure 1-(9H-fluoren-9-yl)-3,14-dioxo-2,7,10-trioxa-4,13-diazaheptadecan-17-oic acid (Fmoc-PEG-COOH) was generously provided by Dr. Christopher Nelson. HPLC solvents consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 9:1 acetonitrile/water). All cancer cell lines were obtained from the NCI-60 repository. Athymic nu/nu mice were obtained from the NCI Animal Production Facility, and utilized for *in vivo* studies following all animal welfare regulations as detailed by the Animal Care and Use Committee (ACUC). All peptides utilized for experiments were prepared with an amidated C-terminus.

2.2 Synthesis of fluorescently-labeled peptides (2)

Synthesis of fluorescently-labeled PEG-GG-SVS-1 conjugates was performed as described in Supplementary Scheme S1. Briefly, resin-bound GG-SVS-1 (GGKVKVKVKV^DPPTKVKVKVK-NH₂), the mirror image enantiomer peptide GG-^DSVS-1, or it's non-folding analogue GG-SVS-2 (GGKVKVKVKVPPTKVKVKVK-NH₂) were synthesized on PL-Rink resin using an automated ABI 433A peptide synthesizer. Synthesis was carried out via Fmoc-based solid-phase peptide chemistry with HCTU activation. On resin, the N-terminal amine of each peptide was reacted with 3 eq. of Fmoc-

PEG-COOH (Fmoc-NH-(CH₂CH₂O)₂(CH₂)₂NHCO(CH₂)₂COOH) in NMP containing DIEA (6 eq.) and HATU (3 eq.) for 6 h at room temperature while shaking to produce each enantiomer of Fmoc-PEG-GG-SVS-1, and Fmoc-PEG-GG-SVS-2. Fmoc-deprotection was then carried out by the addition of 1% DBU and 19% Piperidine in NMP (3 cycles) for 20 minutes per cycle while shaking at room temperature. The resin was washed with CH₂Cl₂ between cycles. Fluorescein(FI)-OSu (2 eq.) and DIEA (4 eq.) in NMP were added to each peptide on resin and reacted at room temperature for 2 h while shaking. Resins were washed with CH₂Cl₂ and dried by lyophilization. Dried resin-bound FI-PEG-GG-SVS-1 (**2**), its enantiomer, and FI-PEG-GG-SVS-2 were cleaved from the resin and simultaneously side-chain deprotected using a trifluoroacetic acid/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2) cocktail for 2 h under argon atmosphere. After filtration, the crude product was precipitated with cold diethyl ether, collected and dried on the lyophilizer. Each peptide was purified by reverse-phase HPLC equipped with a semi-preparative Vydac C18 column. Linear gradients of 0% – 100% solvent B over 100 min. was utilized to purify the peptides. The purity of each peptide conjugate was verified by analytical HPLC and electrospray ionization (ESI-positive mode) mass spectrometry. Analytical HPLC chromatograms and ESI (+) mass spectra for the pure peptides are shown in the Supplementary Figs. S1 and S2.

2.3 Synthesis of PTX-S-S-PEG-GG-SVS-1 (**7**)

The synthesis of (**7**) in Supplementary Scheme S2 entails the coupling of Paclitaxel-2-(2-(pyridin-2-yl)disulfanyl)phenyl)acetate (**6**) with the thiol-functional HS-(CH₂)₂CO-PEG-GG-SVS-1 (**4**) according to the succeeding protocol. The synthesis of (**6**) and its intermediates, as well as the resin-bound intermediates leading to (**4**), is given below.

2.4 Synthesis of HS-PEG-GG-SVS-1 (**4**) and its enantiomer HS-PEG-GG-^DSVS-1

H₂N-PEG-GG-SVS-1 (**1**) or H₂N-PEG-GG-^DSVS-1 on resin was reacted with 3-(tritylthio)propionic acid (3 eq.) in NMP containing DIEA (6 eq.) and HCTU (3 eq.) for 2 h at room temperature while shaking to produce **3**. Dried resin-bound trityl-S(CH₂)₂CO-PEG-GG-SVS-1 (**3**), and its enantiomer, were cleaved from the resin and simultaneously side-chain deprotected using a trifluoroacetic acid/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2) cocktail for 2 h under argon atmosphere. The crude products were precipitated with cold diethyl ether and then dried. Purification was performed by reverse-phase HPLC following the procedure for compound **2**, with fractions collected directly in a round bottom flask on dry-ice to immediately freeze the product. Lyophilization produced (**4**) and its enantiomer as powders.

2.5 2-(2-(pyridin-2-yl)disulfanyl)phenyl)acetic acid (**5**)

In a typical reaction, 2-mercaptophenylacetic acid (100 mg, 0.594 mmol) is dissolved in MeOH (0.5 mL) to which 1 eq. of 1,2-di(pyridin-2-yl)disulfane (131 mg, 0.594 mmol) is added. The reaction was stirred for 4 h at room temperature under Ar, followed by concentration in vacuo. Product was purified on silica gel using an automated flash chromatography system employing a gradient of 0% – 100% ethyl acetate in hexanes over 15 min. yielding 103 mg of compound **5** (0.373 mmol, 63% yield) as a waxy yellow resin. ESI-MS [M+H]¹⁺: calcd 278.0; obsvd 278.1.

2.6 Paclitaxel-2-(2-(pyridin-2-ylidisulfanyl)phenyl)acetate (**6**)

Disulfide **5** (85 mg, 0.373 mmol) was dissolved in CH₂Cl₂ (20 mL) containing Paclitaxel (319 mg, 0.374 mmol) and DMAP (2.3 mg, 0.019 mmol), and cooled to 0°C. DCC (77.2 mg, 0.374 mmol) dissolved in CH₂Cl₂ (4 mL) was added dropwise, after which the reaction was warmed to room temperature and stirred overnight. After concentration in vacuo the product was purified on silica gel using an automated flash chromatography system employing a gradient of 0% – 80% ethyl acetate in hexanes over 20 min. yielding 282 mg of compound **6** (0.253 mmol, 68% yield) as a white crystalline powder. ESI-MS [M+H]¹⁺: calcd. 1114.3, obsvd. 1114.6.

2.7 Final Assembly of PTX-S-S-PEG-GG-SVS-1 (**7**)

In a typical reaction, compound **6** (6.8 mg, 0.006 mmol) was dissolved in DMSO (0.2 mL) to which HS-PEG-GG-SVS-1 (**4**) or HS-PEG-GG-*D*SVS-1 (10.3 mg, 0.004 mmol) was added in portions as a dry fluffy solid, and reaction stirred for 6 h under Ar. The reaction solution was then diluted with DMSO (9.8 mL) and purified by reverse-phase HPLC using a linear gradient of 0% – 30% solvent B over 15 min., followed by 30% – 100% solvent B over an additional 70 min. Collected fractions were combined and lyophilized to yield 3.5 mg of pure **7** (0.001 mmol, 30% yield). The synthesis of PTX-S-S-PEG-GG-*D*SVS-1 is similar. The purity of PTX-S-S-PEG-GG-SVS-1 and PTX-S-S-PEG-GG-*D*SVS-1 was assessed by analytical HPLC and electrospray ionization (ESI-positive mode) mass spectrometry. Analytical HPLC chromatograms and ESI (+) mass spectra for the pure conjugates are shown in the Supplementary Figs. S3 and S4.

2.8 Circular Dichroism Spectroscopy

Circular Dichroism (CD) experiments were performed to determine the secondary structure of the FI-PEG-GG-SVS-1 and PTX-S-S-PEG-GG-SVS-1 in the presence of large unilamellar vesicles (LUV) as model lipid membranes. LUV of POPC and POPC/POPS (1:1) were prepared by extrusion techniques as previously described [1]. CD spectra of the peptide conjugates were obtained in aqueous solution (50 mM BTP, 100 mM KF, pH 7.4) in the absence and presence of LUV (final lipid concentration 2.5 mM). Peptide solutions (50 µM) were used to provide a peptide/lipid ratio of 1:50. Wavelength spectra were measured from 200 nm to 260 nm at 37 °C using a 1 mm path length quartz cell. The mean residue ellipticity, $[\theta]$, was calculated from the equation $[\theta] = (\theta_{\text{obs}}/10lc)/r$, where θ_{obs} is the measured ellipticity (mdeg), l is the length of the cell (cm), c is the molar concentration, and r is the number of residues. CD spectra were collected on an AVIV model 420 circular dichroism spectrometer (AVIV Biomedical, Lakewood, NJ).

2.9 Cellular internalization of FI-PEG-GG-SVS-1

For live cell imaging studies 2×10^4 A549 cells/well were seeded in 4-well chambered glass slides and allowed to adhere overnight under normal culture conditions. Cells were washed with serum free RMPI-1640 media and incubated with 2 µg/mL Hoechst 33342 dye for 20 min. Glass slides were washed with serum free media and mounted onto a Zeiss LSM NLO510 Confocal Microscope with an environmental chamber to maintain 37°C and 5% CO₂ during live cell imaging experiments. Media was then removed from a selected well

and replaced with 1 mL of serum free media containing 5 μ M FI-PEG-GG-SVS-1. Samples were incubated for 5 – 60 min., followed by a final wash and addition of 1 mL blank media before imaging. Cells were visualized using a Neofluar 40 $\times$ oil objective, with planar and z-stack images collected on a selected cell utilizing a two-photon 800 nm laser for the Hoechst signal (band pass emission filter 390 nm – 465 nm) and a single photon 488 nm laser for the fluorescein and phase contrast signals (band pass emission filter 500 nm – 550 nm). Microscopy images were processed using LSM 5 software and shown as the overlaid fluorescein and Hoechst signals, with the borders of the cell outlined in white from the phase contrast signal for clarity. Percentage of total fluorescence at the membrane, within the cytoplasm or localized to the nucleus for each image was determined using ImageJ software and values normalized to background fluorescence. The observed total fluorescence measured in the cytoplasm at each time point was corrected to account for the pH-dependence of fluorescein emission. Briefly, results from the flow cytometry experiments (detailed below) indicate that ~35% of FI-PEG-GG-SVS-1 internalization into A549 cells occurs via endocytosis. Based on the reported pH-dependent fluorescence behavior of dextran conjugated fluorescein probes [3], we determined that approximately 57% of the conjugate's fluorescent intensity was attenuated in the acidic environment of the endosome (pH ~6.0) compared to the cytosol (pH ~7.4). Thus, the observed fluorescent values, and subsequently the reported (rep) fluorescence, in the cytoplasm was corrected for the fraction of conjugate uptake that occurs via endocytosis using the equation: $F_{\text{rep(cytoplasm)}} = F_{\text{obs(cytoplasm)}} + 0.57(0.35 \cdot F_{\text{obs(cytoplasm)}})$.

Flow cytometry studies were performed by seeding 2.5×10^5 A549, HeLa, MCF-7 or HUVEC cells/well in a 24-well plate and allowing the cells to adhere overnight under normal culture conditions. Cells were washed with media before addition of 0.5 mL serum free media containing 2 μ M of FI-PEG-GG-SVS-1 or FI-PEG-GG-SVS-2 peptide, followed by incubation for 1 h. To determine the role of endocytosis on FI-PEG-GG-SVS-1 internalization, cancer cells were depleted of ATP to inhibit general endocytosis following a standard protocol [4]. Briefly, before peptide addition, cells were pre-incubated in glucose and serum free media containing 10 mM sodium azide and 50 mM 2-deoxy-*D*-glucose for 30 min. In a separate experiment, cells were pre-incubated in serum free media containing 0.45 M sucrose for 1 h to selectively prevent clathrin-mediated endocytosis [5]. Samples were then washed with serum free media and FI-PEG-GG-SVS-1 internalization performed as previously described. After treatment, cells were washed with cold PBS and incubated with 150 μ L of 0.25% trypsin-EDTA solution for 15 min. to both collect cells for analysis and digest any non-internalized peptide adsorbed to the surface of the cell based on a previously published protocol [6]. Cells were then pelleted by centrifugation at 2,000 rpm for 5 min. before suspending them in 1 mL of fresh PBS. Samples were analyzed using a Beckman Coulter FACsCalibur flow cytometer (488 nm excitation laser) with gating based on normalized fluorescence of untreated cells to evaluate the percentage of cells which internalized the fluorescently-labeled peptides. Uptake studies were performed in three independent experiments using three replicates for each experimental condition.

2.10 Solubility Assessment for PTX and PTX-S-S-PEG-GG-SVS-1

To determine the increase in PTX solubility upon conjugation to the PEG-GG-SVS-1 carrier, dry powders of PTX or the PTX-S-S-PEG-GG-SVS-1 conjugate were precisely weighed out to afford individual samples, that after adding 100 μL of 25 mM HEPES buffer (pH 7.4), would produce 1 – 1000 μM solutions, if the compounds were fully soluble. The solutions were then shaken for 24 h at 37°C, followed by centrifugal filtration to remove undissolved particulates. Solubilized PTX or PTX-S-S-PEG-GG-SVS-1 was quantified by HPLC using a linear gradient of 0% – 100% solvent B over 100 min., with the area under the curve of the UV signal ($\lambda = 220$ nm) compared to standards prepared in ACN to determine the soluble concentration. Solubility studies were performed in triplicate for each experimental condition. Fold enhancement in PTX solubility when conjugated to the SVS-1 peptide was assessed by comparing the prepared concentration at which free PTX and the conjugate achieved similar percentage solubility values.

2.11 In Vitro PTX Release Studies

PTX release studies were performed by preparing 250 μL of a 0.5 mg/mL (111 μM) solution of PTX-S-S-PEG-GG-SVS-1 in 25 mM HEPES buffer (pH 7.4). A 250 μL solution of 5 mM glutathione in HEPES buffer was added to the PTX-S-S-PEG-GG-SVS-1 solution to initiate conjugate reduction. The solution was shaken at 37°C. At 0, 5, 15, 30 or 60 min. time points, a 65 μL aliquot was removed, mixed with an equal volume of ACN and snap frozen before analysis by HPLC ($\lambda = 220$ nm). Concentration of PTX released was determined by comparing the area under the curve for the free PTX peak to standards prepared in ACN. PTX release studies were performed in triplicate.

2.12 Cytotoxicity of PTX-S-S-PEG-GG-SVS-1 Conjugates

For in vitro cytotoxicity studies, A549 (lung carcinoma), OVCAR-3 (ovarian adenocarcinoma) and NCI/ADR-RES (multi-drug resistant ovarian adenocarcinoma) cells were plated at 5×10^3 cells/well, while MCF-7 (breast adenocarcinoma) cells were plated at 10×10^3 cells/well, in a 96 well plate and allowed to adhere overnight. The culture media was then removed and cells treated with 0.01 – 100 μM concentrations of the SVS-1 peptide, or 0.001 nM – 10 μM concentrations of free PTX or the PTX-S-S-PEG-GG-SVS-1 conjugate in serum-containing media for 72 h. Blank media and 20% DMSO containing media were used as negative and positive controls, respectively. After this incubation period, cells were washed and 100 μL of fresh media was added to each well. 10 μL of MTT solution (5 mg/mL in PBS) was added to each well and samples incubated for 2 h for A549, OVCAR-3 and NCI/ADR-RES cells, or 4 h for MCF-7 cells. The supernatant was then removed from each well and replaced with 150 μL of DMSO to dissolve the formazan product. Absorbance was then read at 540 nm using a UV plate reader (Biotek, Winooski, VT). The absorbance of the positive controls was subtracted from each sample as a blank, and percent viability calculated using the equation: $(\text{Absorbance}_{\text{peptide-treated cells}} / \text{Absorbance}_{\text{untreated cells}}) \times 100$. IC₅₀ values were computed using the Graphpad 5.0 software package and represented as the average of three independent experiments $\pm$ standard deviation.

2.13 Serum Stability of GG-SVS-1 and GG-^DSVS-1 Peptide Carriers

Human blood was collected from healthy volunteers in non-heparin tubes and kept at room temperature for 30 min. to clot. Blood was then centrifuged at $2,000 \times g$ for 10 min. at 4°C, and serum either used immediately or stored as aliquots at -80°C before use. Dry GG-SVS-1 or GG-^DSVS-1 peptides were then dissolved in 600 µL of 1:3 serum:buffer (20mM Tris-HCl, 100mM NaCl, pH 7.4) to a final concentration of 300 µM. At 0, 0.5, 1, 2, 4 or 8 hours a 100 µL aliquot was removed and diluted with an equal volume of 15 wt% TCA and kept on ice for 15 min. The samples were then centrifuged at 13,000 rpm for 10 min. and supernatant analyzed by HPLC. Area under the curve for the intact peptide at each time point was compared to $t = 0$ h to determine the percent of intact peptide as a function of time. As a positive control, Penetratin (RQIKIWFQNRRMKWKK-NH₂) was also studied following the same protocol. Peptide serum stability studies were performed in triplicate for each experimental condition.

2.14 In Vivo Biodistribution Studies

For biodistribution studies, Cy5-labeled peptides were prepared and purified following the protocol described in the synthesis of FI-PEG-GG-SVS-1 (compound **2**), except using a Cy5-NHS ester as the fluorescent precursor. Analytical HPLC chromatograms and ESI (+) mass spectra for the pure peptides are shown in the Supplementary Figs. S5–S7. A549 tumor-bearing mice were prepared by injecting 100 µL of sterile HBSS containing 2×10^6 A549 cells into the right flank of athymic nu/nu mice. Once tumors reached approximately 100 mm³ in volume, a 100 µL solution of Cy5-PEG-GG-SVS-1, Cy5-PEG-GG-^DSVS-1 or Cy5-PEG-GG-NH₂, at 15 µM was administered by tail vein injection. Mice were anesthetized in the induction chamber with 2.5% – 3% isoflurane with 1 L/min flow rate 5 min. prior to the imaging time point. At 0.5, 1, 2, 4, 8 and 24 h after administration mice were transferred to the imaging chambers, isoflurane reduced to 2%, and 2D fluorescence imaging performed using the IVIS Spectrum imager (ex: 640 ± 15 nm and em: 680 ± 10 nm filters; auto exposure; field of view = 19.4 cm). Imager specific Living Image software (version 4.3.1) was used for data acquisition and analysis. To determine the ratio of tumor vs. skin fluorescence, a region of interest was established at the tumor periphery and total fluorescence (measured as radiant efficiency) was compared to a distal region of interest on the skin as the background.

2.15 In Vivo Efficacy Studies

For all in vivo efficacy studies, the GG-^DSVS-1 peptide carrier used to prepare PTX-loaded conjugates were first purified of any contaminating endotoxin before subsequent synthetic steps. This was done by loading 10 mL of a 10 mg/mL peptide solution in sterile water to a high capacity endotoxin removal spin column containing 1 mL of resin (Pierce, Rockford, IL), and shaken for 4 h. After centrifugation following the manufacturer's instructions, the retrieved peptide was then lyophilized and tested for endotoxin levels using a LAL chromogenic endotoxin quantification kit (Pierce, Rockford, IL). Endotoxin levels were routinely found to be <2 EU/mL for the final pure peptides. Before efficacy studies were initiated, the maximum tolerated dose (MTD) of the PTX-S-S-PEG-GG-^DSVS-1 conjugate, prepared in 5% EtOH/95% sterile HBSS solution, was determined in non-tumor bearing

mice ($n = 3$). The maximum soluble concentration of the free PTX drug in this vehicle (1 mg/kg) was also administered to non-tumor bearing mice to monitor toxicity, and found to be well tolerated. All treatment solutions were injected via tail vein at 10 mL/kg volumes. MTD for the PTX-S-S-PEG-GG-*D*SVS-1 conjugate was determined by administering the compound every other day for 7 days, totaling 3 injections. During this period, and for an additional week after treatment, animal body weight measurements and clinical observations of toxicity were performed daily. Dosing of the compound was increased until any single animal showed weight loss greater than or equal to 20%, if they were unable to obtain food or water, had difficulty breathing, or appeared moribund; at which point the dosing level was considered to have exceeded the MTD. From these studies the MTD of the PTX-S-S-PEG-GG-*D*SVS-1 conjugate was established at 20 mg/kg. Tumor burden reduction studies were performed using the same A549 lung cancer mouse model utilized for biodistribution studies, and treatment initiated when tumors reached $\sim 100 \text{ mm}^3$ in volume. Injections were performed 3 times per week for 4 weeks, with animals receiving the PTX-S-S-PEG-GG-*D*SVS-1 conjugate prepared at the MTD in 5% EtOH/95% sterile HBSS, or free PTX dissolved at the maximum soluble dose in the same vehicle ($n = 7$ per group). During treatment, animals were monitored daily for clinical signs, with body weight and tumor growth (caliper) measurements made every other day. Following the final therapeutic injection on day 25, animals were monitored for an additional 12 days to track tumor growth after the cessation of treatment, at which time the tumors in the vehicle and free PTX groups began to ulcerate. The study was terminated at this time. Additionally, during the study period any animal with a tumor dimension larger than 2 cm was euthanized by CO_2 asphyxiation.

3. Results and Discussion

3.1 Cancer Cell-Surface Induced Folding Triggers the Intracellular Translocation of SVS-1

The cell-penetrating ability of SVS-1 was first assessed by conjugating fluorescein to the peptide, and measuring its internalization into A549 lung carcinoma cells (Fig. 2). Fluorescein, a commonly used dye for intracellular tracking, was covalently attached through a PEG linker ($\text{NH}_2-(\text{CH}_2\text{CH}_2\text{O})_2(\text{CH}_2)_2\text{NHCO}(\text{CH}_2)_2\text{CO}-$) at the N-terminus of the peptide (Supplementary Scheme S1). CD spectroscopy studies, employing model vesicles, confirmed that addition of the fluorescent label and PEG linker did not change the folding behavior of the parent SVS-1 peptide (Supplementary Fig. S8). Specifically, the CD spectra of an aqueous solution of FI-PEG-GG-SVS-1 alone, or in the presence of neutral lipid vesicles made of POPC, are indicative of a random coil conformation indicating that the peptide is unfolded in the absence of a negatively charged surface. While, FI-PEG-GG-SVS-1 added to a solution of negatively charged vesicles, made of a 1:1 mixture of POPC/POPS, resulted in a CD signal with a distinct minimum in mean residue ellipticity at 218 nm, indicating that the fluorescently-labeled peptide folds and adopts β -sheet structure at the vesicle's surface. At any rate, Fig. 2 shows live-cell imaging of FI-PEG-GG-SVS-1 added to A549 cells as a function of time (Fig. 2, top). Shortly after the addition of a 5 μM solution of peptide, it localizes as punctate regions of fluorescence primarily on the cell surface, with analysis of the signal indicating that approximately 60% of the peptide is observed at the membrane (Fig. 2, bottom). After this initial binding of the peptide to the cell membrane,

SVS-1 rapidly translocates to the cytoplasm and subsequently penetrates into the nucleus at both 15 and 30 minutes after peptide addition. Finally, at 60 minutes the majority of the peptide fluorescence is observed in the nucleus. These results show that SVS-1 is capable of rapidly traversing the cell membrane, diffusing into the cytoplasm and localizing to the cell nucleus.

The mechanism(s) by which SVS-1 enters cells was investigated using the fluorescein labeled peptide in a panel of cancer cell lines, as well as non-cancerous cells as controls. In general, peptides can enter cells through a number of mechanisms, including physical translocation and various endocytic pathways such as micropinocytosis and clathrin-mediated endocytosis [7–9]. One distinct peptide sequence may use multiple pathways to enter a given cell. Here, a series of flow cytometry studies were performed under different conditions to probe the different internalization mechanisms available to the peptide. Fig. 3A–C, show internalization data for A549, HeLa and MCF-7 cancer cells, respectively. The open bars in each panel show the percentage of cells that have internalized the FI-PEG-GG-SVS-1 peptide after 1 hour incubation. The data show that both A549 and HeLa cells readily uptake the peptide with 95% and 89% of the cells demonstrating fluorescence, respectively. MCF-7 cells also internalize the peptide, but to a lesser extent (52%). Next, general cellular endocytic activity was inhibited by depleting the cellular pool of ATP. This was accomplished by pre-incubating the cells with NaN_3 and 2-deoxy-*D*-glucose [5, 6, 10]. Under these conditions, internalization of SVS-1 in the cancer cells was reduced by 35% – 45% compared to no pre-treatment of cells, indicating that a fraction of SVS-1 cellular uptake is mediated by endocytosis. The contribution of clathrin-dependent and clathrin-independent endocytic pathways to peptide internalization was further probed by selectively inhibiting clathrin-mediated endocytosis before addition of the FI-PEG-GG-SVS-1 peptide. Clathrin-dependant endocytosis occurs through assembly of clathrin into coated pits at the surface of cells that pinch off to form vesicles encapsulating bound ligands [11]. The formation of these vesicles can be blocked by pre-incubating cells in a hyperosmolar (0.45 M) solution of sucrose [5]. Results show that inhibition of clathrin-dependent endocytosis produced a similar reduction in SVS-1 internalization compared to ATP depletion for A549 cells (Fig. 3A), while accounting for approximately half of the reduction in HeLa cells (Fig. 3B) and had no effect in MCF-7 cells (Fig. 3C). This suggests that peptide entering cells via endocytosis does so through a clathrin-dependent mechanism, and that internalization of this pathway varies with cell type. Important to note is that collectively, the data demonstrate that SVS-1 is predominately internalized via physical translocation across the membrane of cancer cells, with a fraction of peptide uptake attributed to mainly clathrin-dependent endocytosis.

The exact mechanism by which SVS-1 enters the nucleus from the cytoplasm is not known. However, interestingly, the composition of the mammalian nuclear and plasma membranes are very similar in regards to lipid content [12]. Therefore, it's probable that engagement of the SVS-1 peptide with phosphatidylserine in the nuclear membrane mediates translocation of the peptide into the nucleus. Further, the time scales over which the SVS-1 peptide crosses the plasma membrane into the cytoplasm (5 – 15 min.), and then from the cytosol to the nucleus (15 – 30 min.), is similar, further supporting this potential mechanism.

The cell penetrating activity of SVS-1 was also found to be preferential for cancer cells, with only 31% of non-cancerous HUVEC cells showing fluorescence after a 1 hour incubation with FI-PEG-GG-SVS-1 (Fig. 3D), in comparison to 95% for A549, as an example. This selectivity is largely driven by the increased electronegative character of the tumor cell surface as compared to non-cancerous cells. Cancer cells are characterized by an increase in phosphatidylserine content in the outer leaflet of the cell membrane [13–15], as well as aberrant glycosylation resulting in increased levels of sialic acid at the cell surface [16–18]. Finally, to determine the influence of peptide folding on the cell penetrating activity of SVS-1, cells were incubated with fluorescently-labeled control peptide, SVS-2 (KVKVKVKV^LPPTKVKVKVK-NH₂). This control peptide contains an ^LPP motif, as opposed to the ^DPP of SVS-1. This simple change in proline stereochemistry prevents the formation of the bioactive β -hairpin conformation, even in the presence of negatively charged membranes [1]. Results show only 3% – 11% of cancerous and non-cancerous cells internalized SVS-2 after a 1 hour incubation, indicating that the folding of SVS-1 at the cell surface is a requirement for membrane intercalation and cellular penetration of this peptide.

3.2 SVS-1 Solubilizes and Delivers Ligated Hydrophobic Drug to Cancer Cells

The live-cell imaging and flow cytometry data show that SVS-1 is capable of rapid cellular internalization, and suggests its utility as a drug delivery vehicle. This possibility was initially explored by investigating the peptide's potential to solubilize a hydrophobic small molecule drug, internalize the drug into cells and release it. Paclitaxel (PTX) was selected as a model chemotherapeutic due to its broad use against a range of solid tumors, including ovarian, breast and non-small cell lung cancers. Currently, clinical administration of hydrophobic PTX involves the use of chemical solubilizing agents, including Cremaphor EL, which are associated with serious toxicities [19], or complexing the drug to nearly stoichiometric amounts of a carrier protein such as albumin [20]. Thus, PTX serves as an excellent model small molecule drug in which to assess the potential of new delivery vehicles. PTX acts on cytoskeletal tubulin in the cytoplasm. Since SVS-1 ultimately localizes to the nucleus via the cytosol, PTX was conjugated to the peptide through a previously reported self-immolative disulfide linker (Supplementary Scheme S2) [21, 22] to ensure its rapid release in the cytoplasm.

Briefly, HS(CH₂)₂CONH(CH₂CH₂O)₂(CH₂)₂NHCO(CH₂)₂CO-GG-SVS-1 was assembled on PAL-amide resin, cleaved, side-chain deprotected and purified. The peptide's free thiol was reacted in solution with Paclitaxel-2-(2-(pyridin-2-yl)disulfanyl)phenyl)acetate to yield Paclitaxel-functionalized SVS-1 after purification, Fig. 1. Conjugation of PTX to SVS-1 resulted in a nearly 1000-fold increase in its solubility in buffer (pH 7.4, 37°C) compared to the free drug (Fig. 4A). This significant increase in solubility is largely due to the eight lysines contained within the SVS-1 sequence, which at physiologic pH are protonated [23]. CD spectroscopy confirmed that conjugation of PTX to PEG-GG-SVS-1 did not prevent folding of the peptide at the surface of phosphatidylserine containing liposomes (Supplementary Fig. S9). Next, the glutathione-mediated release of free PTX from the PTX-S-S-PEG-GG-SVS-1 conjugate was studied. The drug should be released by a glutathione-mediated reduction once internalized into cells, where cleavage of the conjugate's disulfide leads to the self-immolative cyclization of the linker moiety and release of the free drug

(Fig. 1). Drug release kinetics was investigated *ex vitro* by incubating the PTX-S-S-PEG-GG-SVS-1 conjugate in 5 mM glutathione. The concentrations of the released drug, as well as the parent compound, were monitored as a function of time via HPLC (Fig. 4B and Supplementary Fig. S10). Results show that PTX is rapidly released from the PTX-S-S-PEG-GG-SVS-1 conjugate in the presence of physiologically relevant concentrations of glutathione, with 73% of the PTX liberated after 5 minutes of incubation, and quantitative release realized after 120 minutes. Correspondingly, the parent PTX-S-S-PEG-GG-SVS-1 conjugate is completely reduced over the same time period. These results confirm that the PTX-S-S-PEG-GG-SVS-1 conjugate is able to rapidly release the loaded drug upon exposure to intracellular concentrations of glutathione. As a result, this conjugate shows potential to directly deliver PTX to the cytoplasm of cancer cells leading to cancer cell death.

The cytotoxic activity of the PTX loaded SVS-1 peptide was evaluated in A549, MCF-7 and OVCAR-3 cancer cells, as well as a multi-drug resistant NCI/ADR-RES cell line (Fig. 5 and Supplementary Fig. S11). These experiments evaluate the ability of SVS-1 to deliver PTX without influencing the drug's cytotoxic behavior once released. If the peptide acts benignly in its capacity to deliver, one should expect the activity of the PTX-loaded conjugate to be similar to the free drug. When delivered in a DMSO-containing vehicle, free PTX demonstrates low nM IC_{50} values, dependent on cell type [24, 25]. Gratifyingly, treatment of A549 cells with PTX-S-S-PEG-GG-SVS-1 resulted in potent cytotoxic activity (IC_{50} = 7.4 nM), which is on the order of free PTX (IC_{50} = 3.6 nM) as shown in the top panel of Fig. 5. For comparison, the lytic behavior of the free peptide is also shown, affecting its action at much higher concentrations. Similar behavior was observed for the MCF-7 and OVCAR-3 cells, as reported in the table. Interestingly, the peptide conjugate shows slightly enhanced cytotoxicity (10-fold) towards the OVCAR-3 cells as compared to free PTX. The basis for this enhanced activity is not yet known, but might be linked to the high levels of negatively charged heparin sulfate known to exist at the surface of this particular cell type [26], that could enhance uptake of the peptide conjugate.

Lastly, the activity of PTX-S-S-PEG-GG-SVS-1 towards the multi-drug resistant NCI/ADR-RES cell line was assessed. These cells provide a challenging test of the peptide's penchant for delivery. Against this cell line, the PTX-peptide conjugate has an IC_{50} of 4,929 nM, whereas free PTX produced an IC_{50} of 9,701 nM, about a 2-fold decrease in activity compared to the peptide conjugate. Interestingly, incubation of NCI/ADR-RES cells with the parent SVS-1 peptide, void of PTX, produced a cytotoxic response (IC_{50} = 3,211 nM) that is more potent than incubation with either the PTX-S-S-PEG-GG-SVS-1 conjugate or the free drug. Thus the peptide's lytic action is more potent than the action of PTX for this cell line. Overall, the cell-based data demonstrates that SVS-1 is capable of effectively delivering PTX to cancer cells.

It should be noted that premature cleavage of disulfide containing conjugates has been recently reported to occur at the surface of cancer cells [27], mediated by secreted thiols and cell surface proteins [28]. The kinetics of this extracellular cleavage is highly dependent on conjugate size and residence time at the membrane, steric and electrostatic microenvironment around the disulfide bond, and the cell line being tested. However, in

general the reduction of disulfides at the cell membrane is significantly slower ($t_{1/2}$ of 1 – 2 h) when compared to rates of disulfide cleavage in the highly reductive intracellular environment. Nevertheless, for our conjugate it cannot be ruled out that a small fraction of ligated PTX may be released at the surface of cancer cells over the short time period (5 – 15 min.) which SVS-1 resides at the membrane (Fig. 2). This, in combination with drug efflux mechanisms, may explain why PTX delivered to NCI/ADR-RES cells via the SVS-1 peptide was not able to achieve the nanomolar activity realized for the sensitive cell lines.

3.3 The SVS-1 Carrier is Stable in Serum

Before initiating the in vivo studies, the serum stability of the SVS-1 delivery vehicle was measured. Here, an SVS-1 derivative (GG-SVS-1) containing two N-terminal glycines was employed to better represent the entire peptidic portion of the PTX-conjugate ultimately used in vivo. Stability measurements were conducted as a function of time using serum taken from healthy, unmedicated human volunteers (Fig. 6). Incubation of GG-SVS-1 in a 25% serum solution for 8 hours resulted in 60% of the peptide being proteolytically degraded, with a corresponding serum half-life of 5.9 hours. The persistence of GG-SVS-1 under these conditions was surprising given the rapid proteolysis reported for other lysine and arginine containing cationic *L*-peptides. For example, the TAT (YGRKKRRQRRR-NH₂) and KSLK (KKVVFVKVFKK-NH₂) peptides have reported half-lives of 3.5 min.[29] and 6 min.[30] in human plasma and mouse serum, respectively. One explanation for the stability of the GG-SVS-1 peptide may be due to the *D*-proline residue contained within its turn region. It is well established that incorporation of non-natural *D*-amino acids into peptide sequences imparts enhanced stability to proteolysis [31]. In order to further increase the serum stability of the peptide, we prepared its enantiomer, which contains nearly all *D*-residues (except for the lone *L*-proline in its turn region). We have previously shown that the *D*-enantiomer also folds at the surface of negatively charged vesicles [1] and should be just as capable of penetrating into cells. Gratifyingly, Fig. 6 shows that 95% of the GG-*D*SVS-1 peptide is intact after an 8 hour incubation in the human serum solution. As a control, the degradation of Penetratin (RQIKIWFQNRRMKWKK-NH₂), which is a widely studied CPP with similar amphiphilicity and formal charge (+8) to the SVS-1 peptide at neutral pH, was also measured under these conditions. In the presence of the 25% serum solution, Penetratin was rapidly reduced with a half-life of approximately 20 min. Since many peptides are limited in their clinical translation as a result of their rapid proteolysis in vivo [32], the intrinsic stability of the SVS-1 peptide, and its enantiomer, makes these attractive carriers for therapeutic applications.

3.4 SVS-1 Conjugates Localize to Tumor Tissue and Elicit Potent Anticancer Activity In Vivo

In vivo biodistribution studies were performed to investigate the ability of the SVS-1 peptide to localize to tumor tissue. Fluorescence was used to measure the distribution of a Cy5-labeled SVS-1 construct (Cy5-PEG-GG-SVS-1) over 24 hours following its i.v. administration to mice bearing A549 lung carcinoma xenografts. Tumor vs. skin fluorescence ratios were monitored as a function of time (Fig. 7). Following the injection of Cy5-PEG-GG-SVS-1, tumor vs. skin fluorescence ratios reached a maximum of approximately 2.3 within 2 hours of administration, as shown in panel A. This initial wash-

in of the peptide was followed by a steady decline in tumor-associated fluorescence, reaching background levels at 24 hours. Thus, the peptide reaches the tumor where it stays localized over about a day. We hypothesized that the residence time of the peptide at the tumor could be increased by limiting its susceptibility to proteolysis. This was confirmed by assessing the tumor-specific retention of the enantiomeric peptide, Cy5-PEG-GG-*D*SVS-1. Panel B shows that Cy5-PEG-GG-*D*SVS-1 washed into tumor tissue over about 2 hours, reaching a tumor vs. skin ratio of around 2.4. Importantly, the enantiomeric peptide resides at the tumor over the full 24 hour time period. Finally, the tumor localization of a control dipeptide (Cy5-PEG-GG-NH₂) lacking the SVS-1 sequence is shown in panel C. The control washed into tumor within an hour after administration, but quickly washes out, suggesting the SVS-1 peptide is necessary for increased tumor residence.

Based on the more favorable distribution and retention profile of the enantiomeric Cy-PEG-GG-*D*SVS-1 conjugate, as well as the excellent serum stability of its peptidic portion, we synthesized the corresponding enantiomeric PTX conjugate (PTX-S-S-PEG-GG-*D*SVS-1) and evaluated its efficacy in vivo. The maximum tolerated dose (MTD) of the conjugate was first evaluated in non-tumor bearing mice by delivering increasing amounts of the peptide conjugate over a one week period, using morbidity as an endpoint. For these studies, the conjugate was administered by tail vein injection in Hank's buffered salt solution (HBBS) containing 5% EtOH. Of note, greater than 3 mg/mL (0.9 mM equivalent PTX) could be solubilized without the need of an adjuvant vehicle such as Cremophor EL or albumin, as compared to only 0.1 mg/mL (0.1 mM) of free PTX. For these studies, we chose not to include a solubilizing agent for administration of free PTX to provide a direct comparison of the therapeutic efficacy of each compound administered under equivalent conditions. The MTD of the PTX-S-S-PEG-GG-*D*SVS-1 conjugate was determined to be 20 mg/kg. Concentrations exceeding this value were toxic probably as a result of the peptide's lytic behavior at high concentrations. Although PTX was only used as a model small molecule drug in our study, its noteworthy that the MTD of the SVS-1 conjugate corresponds to an equivalent PTX dose of 4.9 mg/kg, while the tolerable limit of albumin-bound PTX (30 mg/kg)[20] represents a 0.4 mg/kg dose of free PTX. Thus, a greater than 10-fold increase in the deliverable dose of PTX is achieved when the drug is conjugated to the SVS-1 peptide, as opposed to being complexed to albumin.

Efficacy was measured via tumor reduction studies employing A549 tumor-bearing mice. A549 cells were introduced into the flanks of mice and tumors were allowed to reach ~ 100 mm³, at which time treatment began. Fig. 8A shows percent volume change for mice receiving PTX-S-S-PEG-GG-*D*SVS-1, free PTX, or EtOH/HBSS vehicle, all of which were administered by tail-vein injection. Mice were dosed according to the regimen outlined in Fig. 8A for a total of 25 days, and the percent change in tumor volume was measured every other day. Mice receiving the PTX-S-S-PEG-GG-*D*SVS-1 conjugate had a significantly slower rate of tumor growth compared to animals given free PTX or the vehicle control. Furthermore, after treatment was terminated, the mice dosed with PTX-S-S-PEG-GG-*D*SVS-1 continued to exhibit a slow tumor growth profile, resulting in a 2-fold reduction in final percent tumor volume observed for animals receiving the conjugate compared to administration of PTX or the vehicle. These results suggest the anticancer activity of the

PTX-S-S-PEG-GG-^DSVS-1 conjugate was sustained even after the course of treatment had ended. Importantly, administration of free PTX did not produce a statistically significant reduction in tumor growth rate compared to the vehicle control, indicating that the maximum soluble concentration of the free drug was not sufficient to produce an effective anti-tumor response. These results are further supported through visual observation of tumor tissue excised from mice 12 days after the termination of treatment (Fig. 8B). In these images tumors removed from mice treated with the PTX-S-S-PEG-GG-^DSVS-1 conjugate were found to be significantly smaller than tumors excised from mice receiving PTX alone or the vehicle control. Finally, treatment of tumor-bearing mice with PTX-S-S-PEG-GG-^DSVS-1, free PTX or the vehicle control did not result in any significant loss of animal body weight over the study period (Fig. 8C), indicating all of the compounds were non-toxic at the administered doses. Collectively, these results demonstrate that the ^DSVS-1 peptide can dramatically enhance the solubility of PTX, and effectively deliver the drug to tumor, reducing tumor burden in mice.

4. Conclusions

The ability of SVS-1 and its enantiomer to selectively penetrate cancer cells, their high water solubility, resistance to proteolysis, and demonstrated ability to deliver Paclitaxel, suggests that they will be broadly useful in the delivery of other small hydrophobic molecules. SVS-1 compliments a pioneering class of peptidic carriers with cell penetrating capabilities [33–44], especially those whose activity can be triggered as a function of their local environment [45, 46]. Future studies are focused on reducing the lytic behavior of these peptides to increase their therapeutic index and further explore their potential to deliver molecules orphaned in drug discovery campaigns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Design of the SVS-1-Paclitaxel conjugate. SVS-1 peptide (green) is conjugated to paclitaxel (PTX) (red) through a PEG spacer (blue) and self-immolative di-sulfide linker (purple). The PTX-S-S-PEG-SVS-1 conjugate electrostatically engages the negatively charged surface components of cancer cells, promoting folding of the peptide into a bioactive β -hairpin. This folding event allows the peptide carrier to interpolate into the lipid bilayer leading to translocation of the conjugate into the cancer cell cytoplasm. Intracellular glutathione then reduces the self-immolative disulfide linkage leading to release of free PTX and cancer cell death.

Fig. 2.

(Top) Live-cell imaging of A549 cells after addition of 5 μ M fluorescein-labeled SVS-1 peptide, as a function of time (scale bar = 10 μ m). Associated z-stacks are also shown.

(Bottom) Percentage of total cellular fluorescence measured at the membrane, within the cytoplasm or localized at the nucleus.

Fig. 3.

Internalization of FI-PEG-GG-SVS-1 into (A) A549, (B) HeLa or (C) MCF-7 cancer cells, as well as (D) non-cancerous HUVECs after 1 hour incubation with SVS-1 alone (open bars), or with ATP depletion (gray bars) and hyperosmolar sucrose (hatched bars) pre-conditioning, as well as the control peptide FI-PEG-GG-SVS-2 (black bars). Uptake results under ATP depletion and hyperosmolar sucrose conditions were compared to direct SVS-1 incubation (open bars) to determine statistical significance, which is denoted by ** indicating $p < 0.01$.

Fig. 4.

(A) Enhancement of Paclitaxel (PTX) solubility upon conjugation to the SVS-1 peptide (PTX-S-S-PEG-GG-SVS-1; ●) compared to free PTX (□) after a 24 hour incubation in 25mM HEPES buffer (pH 7.4, 37°C). (B) Release of free PTX (□) and corresponding disappearance of PTX-S-S-PEG-GG-SVS-1 (●) on incubation of the conjugate with 5 mM glutathione at 37°C. Lines included to aid visualization.

Treatment	IC ₅₀ [nM]			
	A549	MCF-7	OVCAR-3	NCI/ADR-RES
PTX-S-S-PEG-GG-SVS-1	7.4 ± 3.6	5.6 ± 2.9	0.1 ± 0.1	4,929 ± 648
PTX	3.6 ± 1.5	1.5 ± 0.8	2.8 ± 0.8	9,701 ± 730
SVS-1	6,157 ± 674	6,811 ± 671	2,442 ± 645	3,211 ± 658

Fig. 5. Representative cytotoxicity profile of the PTX-S-S-PEG-GG-SVS-1 conjugate, free PTX or the SVS-1 peptide towards A549 (lung carcinoma) cancer cells after 72 hours of incubation. GraphPad Prism 5 software was used to fit cytotoxicity curves and, as shown in the table for all the cell lines investigated, calculate IC₅₀ values employing a log(inhibitor) vs. response (three parameter) non-linear regression model.

Fig. 6. Stability of the GG-SVS-1 (○) or GG-^DSVS-1 (◆) carriers, and the control Penetratin (○), in the presence of human serum.

Fig. 7. Time dependent tumor vs. skin fluorescence ratio following i.v. injection of the fluorescently-labeled Cy5-PEG-GG-SVS-1 peptide, its enantiomer Cy5-PEG-GG-^DSVS-1, or the control compound Cy5-PEG-GG-NH₂, to A549 tumor bearing mice.

Fig. 8.

(A) Percent tumor volume change for mice receiving the PTX-S-S-PEG-GG-*D*SVS-1 peptide-drug conjugate at the MTD (20 mg/kg), free drug (PTX) at its maximum soluble dose (1 mg/kg), or vehicle (5% EtOH, 95% HBSS). Red arrows represent the dosing regimen. Results shown as the mean $\pm$ standard error of the mean; * = $p < 0.001$ compared to the vehicle control. (B) Representative images of tumor tissue from mice receiving the PTX-S-S-PEG-GG-*D*SVS-1 peptide-drug conjugate (top), free PTX (middle), or vehicle control (bottom) (scale bar = 0.5 cm). (C) Change in percent body weight of tumor-bearing mice during treatment.