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Targeted Delivery of Paclitaxel to Tumor Cells: Synthesis and in Vitro Evaluation

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We previously reported a novel drug delivery system, drug-linker-Phe-Phe-Arg-methylketone (FFR-mk)-factor VIIa (fVIIa). The method utilizes tissue factor (TF), which is aberrantly and abundantly expressed on many cancer cells. The advantage of this delivery system is its ability to furnish a potent anticancer drug specifically to the tumor vasculature and cancer cells. In this paper, we describe the synthesis of paclitaxel (PTX)-Phe-Phe-Arg-chloromethyl ketone (FFR-ck), followed by coupling with fVIIa to form PTX-FFR-mk-fVIIa. FFRck was separately linked to the OH groups at the C2′ or C7 positions of PTX (C2′- or C7-PTX-FFRck), the C2′ analogue exhibiting better activity against human head and neck squamous KB 3-1 cells. The activity order against PTX-sensitive KB 3-1 cells is C2′-PTX-FFRmk-fVIIa > PTX > C2′-PTX-FFRck. The C2′ complex shows an IC_{50} of 12 nM against the PTX-sensitive cell line and 130 nM against PTX-resistant cells.

Introduction

Target-specific drug delivery has become an important strategy to improve the selectivity of cytotoxic drugs against targeted cells and reduce toxicity against normal cells. The general method couples a drug carrier and the drug to form a prodrug, which can be released in the target cells. Then, the drug is transformed from an inactive to an active form that performs the cell-killing function. Cancer chemotherapy is one of the important approaches to treating cancer patients, although the nonselectivity of many antitumor agents can cause serious side effects. Paclitaxel (PTX) is a mitotic inhibitor that stabilizes microtubules, thereby interfering with the normal dissociation of microtubules during cell division.[1] The compound is widely used to treat patients with lung, ovarian, breast, head, and neck cancer and advanced forms of Kaposi's sarcoma.[2,3] However, serious drawbacks hamper PTX's[a] clinical usefulness. For instance, paclitaxel lacks selective cytotoxicity between cancer cells and normal cells, which frequently leads to serious unwanted side effects.[4] The poor water solubility of paclitaxel is another problem that significantly reduces its wider clinical application. Different strategies have been explored to circumvent these side effects. One of the most attractive tactics is the selective targeting of tumor cells over normal cells exemplified by employing suitable monoclonal antibodies.[5−7] A number of antigens that are preferentially expressed on tumor cells have been identified, and monoclonal antibodies have been developed to specifically bind to these tumor associated substances. Hyaluronan, a linear polysaccharide whose receptors are overexpressed in some tumors, has also been used to target paclitaxel to tumor cells.[8] Additionally, Zhao et al. have demonstrated the targeting of tumor cells using tripeptide modified liposomes that home on integrin receptors overexpressed on tumor cells.[9] Cancer cells are also known to overexpress GLUT, a family of membrane proteins, to improve glucose uptake. Chen et al. have synthesized four glycan-based paclitaxel prodrugs to specifically target GLUT overexpressing cancer cells.[10] Inorganic nanoparticles have also been widely used for imaging, targeting, and drug delivery.[11] Gold nanomaterials represent one approach in which the ability to control the size and shape of the particles and their surface conjugation with antibodies allows for both selective imaging and photothermal killing of cancer cells by using long-wavelength light for tissue penetration.[12−14]

Angiogenesis is a crucial process for tumor progression and metastasis.[15] An emerging strategy in cancer treatment is to target tumor-associated vascular endothelial cells (VECs) aimed at impeding the growth and survival of solid tumors by eliminating tumor blood supply. Tissue factor (TF) is aberrantly overexpressed on tumor VECs and on cancer cells in many malignant tumors, but not on normal VECs, which makes it a favorable target for cancer therapy. Several laboratories have reported promising results by targeting TF in tumor cells and VECs and thereby suppressing tumor growth in animal models.[16−18] Tissue factor is a transmembrane receptor for an endogenous ligand coagulation factor VIIa (fVIIa). Upon binding to fVIIa, TF forms a high-affinity and specific complex with fVIIa, and the complex subsequently initiates the blood clotting sequence.[19] Several tripeptide chloromethyl ketones have been demonstrated to inactivate fVIIa by binding histidine in the serine protease core and render it a competitive inhibitor of unligated fVIIa.[20] Banner and co-workers have reported the crystal structure of fVIIa-TF. The fVIIa component adopts an extended

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[a] Abbreviations: fVIIa, factor VIIa; TF, tissue factor; FFRck, Phe-Phe-Arg-chloromethylketone; FFR-mk, Phe-Phe-Arg-methylketone; PTX, paclitaxel; VECs, vascular endothelial cells; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells; NF-κB, nuclear factor kappa B; NR, Neutral Red.

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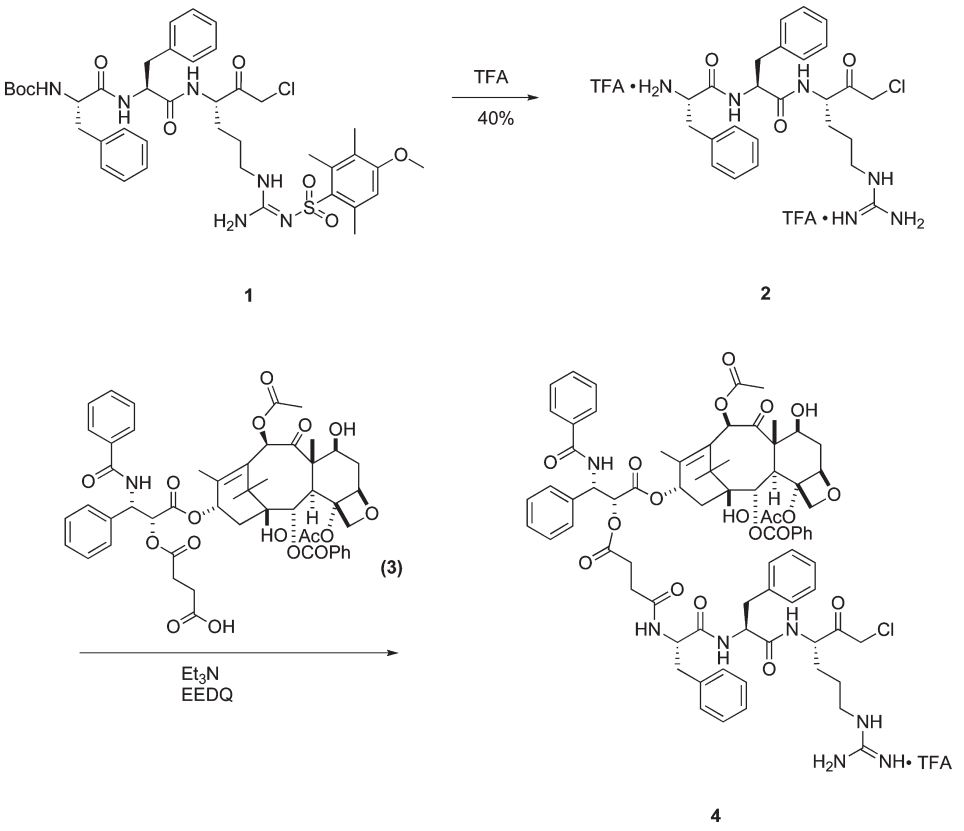
Scheme 1



conformation that wraps around TF with the catalytic domain of the serine protease distal to the cell membrane.[21] Cancer patients are generally in a hypercoagulable state because cancer cells express TF, which is the cognate receptor of fVIIa. FFRck binds and inactivates fVIIa to make it a competitive inhibitor of fVIIa. This is expected to prevent potential patients from thrombotic complications when PTX-FFRmk-fVIIa is administered. It requires coupling of a tripeptide chloromethyl ketone (e.g., FFRck) to the protein. The active site inactivated fVIIa retains the same binding affinity as fVIIa to its receptor protein, TF.[22] On the basis of this evidence, we have chosen FFRmk-fVIIa as a drug carrier with two purposes. First, we intend drugs for targeted drug delivery to tumor angiogenesis and the tumor itself because TF is induced to aberrantly express in cancer VEC by vascular endothelial growth factor (VEGF), and cancers express TF. Second, this should also prevent or minimize an increased tendency of blood clotting in cancer patients.

The use of peptide chloromethyl ketones as the coupling reagent to generate irreversible inhibitors of serine proteases was pioneered by Shaw and co-workers.[23] Our group has previously demonstrated the utility of using fVIIa as a selective anticancer drug carrier. To validate the concept, the cytotoxic agent EF24 was conjugated to fVIIa through a tripeptide-chloromethyl ketone linker.[24] The experiment successfully demonstrated that the conjugate inhibits vascular endothelial growth factor-induced angiogenesis in rabbit cornea and Matrigel models in athymic nude mice.[25] The potential of this particular delivery method can be maximized by inserting highly potent, but highly toxic anticancer drugs, for instance, paclitaxel, into the tumor cells specifically to reduce toxic side effects. In this paper, we describe the synthesis of PTX-FFRck and its conjugation to fVIIa to give PTX-FFRmk-fVIIa. We expected the latter to exhibit behavior similar to EF24-FFRmk-fVIIa, namely specific targeting of cancer cells, but with significantly greater potency.

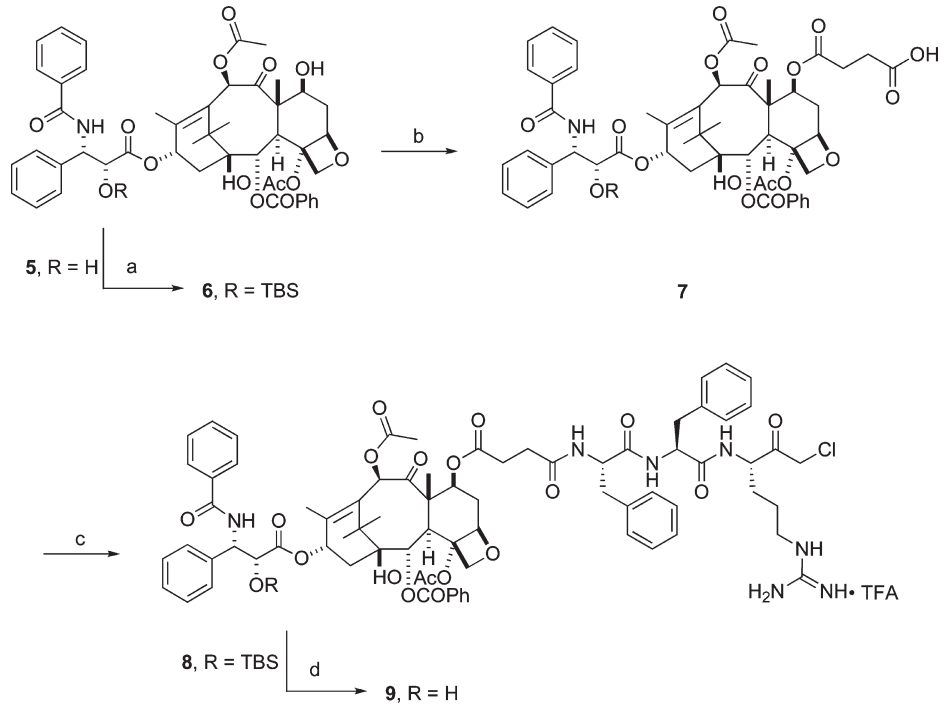
Results and Discussion

The synthesis of C2′-PTX-FFRck is based on the observation that paclitaxel can be esterified at C2′ without protection of other functional groups. The preparation began with the global deprotection of tripeptide **1**[20] to furnish the tripeptide chloromethylketone **2**. The C2′ succinic acid derivative of PTX,[26] namely compound **3**, was treated with EEDQ[27] and then coupled with **2** to afford the desired C2′-PTX-FFRck, **4**, as a TFA salt. (Scheme 1)

To explore the possible biological differences between C2′-FFRck and C7-FFRck, tripeptide chloromethyl ketone (FFRck) was also coupled with the C7-position hydroxyl group of PTX as illustrated in Scheme 2. The C2′-hydroxyl group of PTX was selectively protected with TBSCl in the presence of imidazole to obtain **6**,[28] which was combined with succinic anhydride to give coupling precursor **7**. The conjugation of acid **7** to the TFA salt of FFR-ck **2** yielded **8**. Deprotection of the TBS group under acidic conditions afforded the TFA salt of C7-PTX-tripeptide chloromethyl ketone **9**.

Conjugation of PTX-FFRck to fVIIa. The procedure is essentially the same as that described for conjugating EF24.[21] Briefly, C2′-PTX-FFRck in 100% DMSO is added dropwise to factor VIIa solution in a molar ratio of 3:1 at room temperature for 1−2 h and then gently stirred at 4 °C overnight. The unconjugated excess C2′-PTX-FFRck is removed by dialysis in 10 mM Tris-HCl, pH 7.5, with several

Scheme 2[a]



[a] Reagents and conditions: (a) TBSCl, imidazole, 92%; (b) succinic anhydride, Et_3N, 86%; (c) EEDQ, **2**, Et_3N, 46%; (d) HCl, MeOH, 69%.

changes of the buffer. The resulting C2′-PTX-FFRmk-fVIIa is a competitive inhibitor of fVIIa because PTX-FFRck binds the core of serine protease of fVIIa but retains the same binding activity as fVIIa to TF.[22] The fVIIa activity of the PTX-FFRmk-fVIIa is less than 4−5% of fVIIa as determined according to the manufacturer's instruction using an ST art 4 device to measure factor VIIa coagulation activity (Diagnostica Stargo, Asnieres-sur-Seine, France). The result indicates that approximately 95% of fVIIa is bound by C2′-PTX-FFRck to make C2′-PTX-FFRmk-fVIIa.

Neutral Red Dye Cell Viability Assays for Testing Drug Activity. The activities of C2′- and C7-PTX-FFRck at various concentrations were assessed against KB 3-1 head and neck cancer cells and compared with PTX. Both C2′ and C7 analogues decrease the viability of cancer cells in a dose-dependent manner, although the former is more active than the latter. The cytotoxicities of both PTX-analogues were less than that of free paclitaxel. The IC_{50} of C2′-PTX-FFRck is approximately 12 nM, whereas that of C7-analogue is 130 nM. Thus, the cytotoxic activity of the C2′ compound is approximately 10-fold superior to that of the C7 variation. This undoubtedly arises because the C2′ ester bond is more susceptible to hydrolysis and release of PTX by comparison with the C7 ester (Figure 1).[29] Therefore, C2′-PTX-FFRck (**4**) was chosen for conjugation with fVIIa to form C2′-PTX-FFRmk-fVIIa (abbreviated as PTX-FFRmk-fVIIa, herein).

Activity of PTX-FFRmk-fVIIa in Vitro against PTX-Sensitive and PTX-Resistant Cell Lines. PTX-sensitive (KB 3-1 and TU212) and PTX-resistant (KB-V1) cells were treated with varying concentrations of PTX, C2′-PTX-FFRck, and C2′-PTX-FFRmk-fVIIa. The rank order of activities against PTX-sensitive KB 3-1 and TU212 cells is C2′-PTX-FFRmk-fVIIa > PTX > C2′-PTX-FFRck. The apparent IC_{50} values of C2′-PTX-FFRmk-fVIIa, PTX, and C2′-PTX-FFRck against KB 3-1 cell lines are 0.6, 1.2, and 12 nM, respectively (Supporting Information Figure 1SA and 1SB). PTX, PTX-FFRmk-fVIIa, and PTX-FFRck all show a weaker effect on the PTX-resistant cell line KB-V1 by comparison with the PTX-sensitive cell lines. (Supporting Information Figure 1SC).

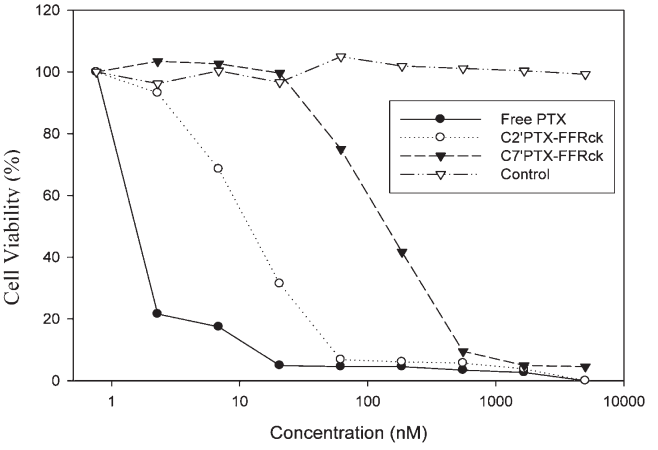


Figure 1. Cell viabilities for C2′-PTX-FFRck and C7-PTX-FFRck. Varying concentrations of C2′-PTX-FFRck, C7-PTX-FFRck, and PTX were incubated with human head and neck cancer cells (KB 3-1) in triplicate for 72 h. Cell viability was determined using the Neutral Red dye assay.

Activity of PTX, PTX-FFRck, and PTX-FFRmk-fVIIa on Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were cultured in the presence or absence of TPA (phorbol ester 100 nM) overnight in order to induce TF because normal HUVECs do not express this protein. HUVECs were incubated with varying concentrations of PTX-FFRmk-fVIIa, PTX, PTX-C2′-FFRck, or PTX-C7-FFRck for 72 h, and then cell viability was determined by Neutral Red dye assays in triplicate. TPA-treated HUVECs express TF, PTX-FFRmk-fVIIa binds this protein, the TF-fVIIa

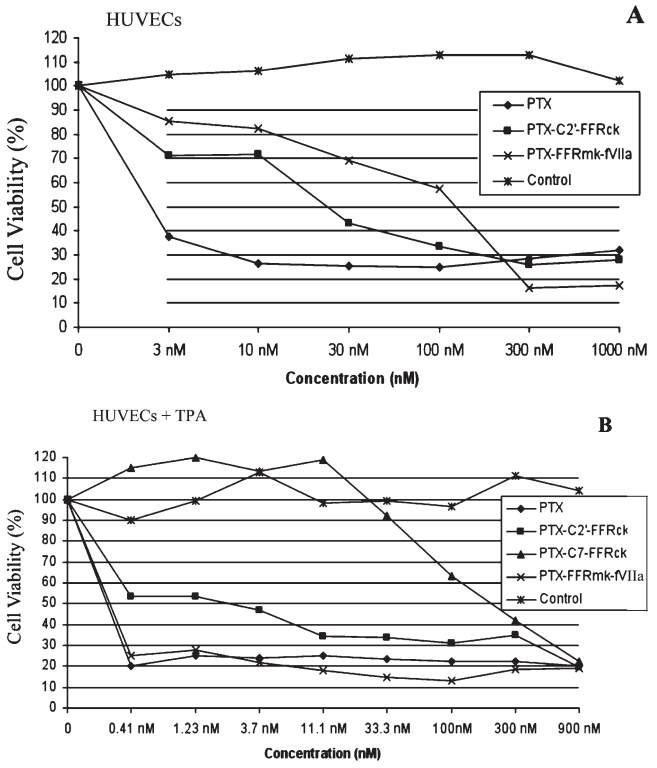


Figure 2. (A) Cell viabilities for PTX, C2′-PTX-FFRck, and PTX-FFRmk-fVIIa on HUVECs. (B) Cell viabilities for PTX, C2′-PTX-FFRck, C7-PTX-FFRck, and PTX-FFRmk-fVIIa on TPA-treated HUVECs.

complex is internalized (endocytosed), and the corresponding HUVECs were killed. By contrast, normal HUVECs do not express TF preventing PTX-FFRmk-fVIIa from binding and subsequent cell kill. PTX alone does not require ligand−receptor-mediated endocytosis to enter cells. Thus, PTX kills both normal HUVECs and TPA-treated HUVECs because it diffuses into the cells and suppresses microtubule dynamics. Moderate cell killing capability was detected for C2′-PTX-FFRck on TPA treated HUVEC cells, which is consistent with our hypothesis that C2′ ester bond is more susceptible to hydrolysis and release of free PTX by comparison with the C7 ester (Figure 2).

We describe here the synthesis of both C2′- and C7-PTX-substituted PTX-FFRcks using succinic acid as a linker between paclitaxel and FFRck. Both paclitaxel and the conjugates exhibit cytotoxic action against the KB 3-1 head and neck cancer cell line. Certain substitutions of the C2′-hydroxyl functionality of the prodrug are known to cause reduction in cyctotoxic activity.[30−32] The C2′-conjugate is ca. 10-fold more active than the corresponding C7-conjugate, which is due to the C2′ ester bond being more susceptible to hydrolysis and release of PTX by comparison with the C7 ester. Paclitaxel itself is slightly more potent than both conjugates against the same cell line. Although esterifying the C2′-hydroxyl group of paclitaxel with succinic acid appeared to slightly reduce cancer cell toxicity relative to free paclitaxel, the linker preserves the activity of paclitaxel and was therefore chosen to implement the coupling chemistry with fVIIa. Subsequently, the paclitaxel sensitive KB 3-1 and TU212 cell lines and the paclitaxel resistant KB-V1 SCC cell line were treated with PTX, C2′-PTX-FFRck and C2′-PTX-FFRmk-fVIIa. The apparent IC_{50} of the fVIIa-conjugate, C2′-PTX-FFRmk-fVIIa, is approximately 30-fold lower than that of the C2′-PTX-FFRck against the TF-expressing cells. The results are consistent with cell surface paclitaxel−conjugate fVIIa binding to TF to deliver a complex subsequently endocytosed by the target. Once inside the cells, it is hypothesized that the PTX-FFRmk-fVIIa complex is enzymatically cleaved to free paclitaxel, which is locally available for cytotoxic action by ablating the dynamic properties of microtubules during cell division. The strategy of using the tripeptide (Phe-Phe-Arg)-ck as a linker and fVIIa as a vehicle for target delivery of highly potent and toxic drugs (e.g., paclitaxel) specifically to cancer cells, further demonstrates the utility of the previously described drug-targeting concept. This delivery mechanism opens a useful approach to reducing the side effects of chemotherapy treatment. Further research in our laboratory is directed toward more efficient methodologies to deliver paclitaxel and other highly toxic anticancer drugs to critical sites of action.

Conclusion

Conjugation of FFRck separately with the C2′-position and C7-hydroxyl groups of paclitaxel leads to the successful preparation of C2′-PTX-FFRck and C7-PTX-FFRck, respectively, in fairly good yields by means of a short synthetic procedure. The C2′ derivative exhibits considerably better activity against KB 3-1 head and neck cancer cells than the C7 analogue. Thus, C2′-PTX-FFRck was coupled with fVIIa using known methods. Cell viability assays for paclitaxel, C2′-PTX-FFRck, and C2′-PTX-FFRmk-fVIIa employing paclitaxel-sensitive and -resistant cancer cells demonstrate two important features: (1) PTX and C2′-PTX-FFRmk-fVIIa are nearly equally efficient for cell killing of paclitaxel sensitive cancer cells in vitro, while C2′-PTX-FFRck is significantly less active. Intuitively, it might be imagined that the active transport of C2′-PTX-FFRmk-fVIIa in complex with TF should be faster than the corresponding passive diffusion of free paclitaxel across the cell membrane and, thereby, occasion higher potency for the protein conjugate. However, the apparent IC_{50} values estimating a diminutive 2-fold advantage for the complex suggests that the two transport mechanisms may be competitive. The important difference, of course, is that while hydrophobic PTX can diffuse across almost all membranes with equal ease, the complex will be actively transported only across cell membranes rich in overexpressed TF. The latter is aberrantly expressed in the most malignant cancers because nuclear factor kappa B (NF-κB) is constitutively activated, while normal cells do not express TF. We previously demonstrated a proof of principle that VEGF attracts VECs and induces TF on VECs. An anticancer reagent, namely EF24-FFRmk-fVIIa, binds to TF expressed on VECs and tumors in vivo, endocytoses, releases the drug, and inhibits TF-expressing VECs and cancer cells in vivo.[25] In the present work, we applied this concept by treatment of cancer cells (KB 3-1 head and neck cancer cells), HUVECs and TPA+ HUVECs with PTX, PTX-FFRck and PTX-FFRmk-fVIIa, respectively. (Figures 1 and 2) These experiments further support our hypothesis on the targeted drug delivery to TF-expressing VECs and tumor cells. The minimal effect observed in vitro will be maximized in vivo under conditions of rapid blood flow. In this way, the specific cell-surface targeting character of C2′-PTX-FFRmk-fVIIa offers promise for both therapeutic efficacy and reduction of side

effects in the living organism. (2) Paclitaxel and the conjugates (C2′-PTX-FFRck and PTX-FFRmk-fVIIa) are all much less effective against the paclitaxel resistant KB-V1 cell line by comparison with treatment of paclitaxel sensitive cell lines.

Experimental Section

General. All amino acids were obtained from Chem Impex International, while all solvents and other reagents were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received. Thin layer chromatography (TLC) was performed on precoated, glass-backed plates (silica gel 60 F_{254}; 0.25 mm thickness) from EM Science, and the plates were visualized by UV lamp. Column chromatography was performed with silica gel (230−400 mesh ASTM) using the "flash" method. Elemental analyses were carried out by Atlantic Microlab Inc. Norcross, GA. All reactions were performed under anhydrous nitrogen atmosphere in oven-dried glassware.

Synthesis of phe-phe-arg trifluoroacetic acid salt (2). Boc-phe-phe-arg (Mtr) chloromethyl ketone, **1**[20] (390.0 mg, 0.480 mmol), was dissolved in TFA (5.0 ML). Thioanisole (200 μL) and water (200 μL) were added and the mixture stirred at RT for 36 h. TFA was removed under vacuum and the crude product washed with ether and then ethyl acetate. The resultant solid was washed with dichloromethane to give **2** (150.0 mg, 43% yield) as a white solid. MS calcd for $C_{25}H_{33}ClN_6O_3$, 500.2303; found, 501.2370 $[M + H]^+$.

Synthesis of 2′-succinyl PTX-phe-phe-arg chloromethyl ketone, C2′-PTX-FFRck, (4). To a solution of C2′-succinyl paclitaxel, **3** (34.0 mg, 0.0357 mmol), in CH_2Cl_2 (500 μL) was added EEDQ (11.0 mg, 0.0445 mmol) and Et_3N (6.0 μL, 0.043 mmol) and the mixture stirred for 30 min. To the solution was added Et_3N (18.0 μL, 0.043 mmol), FFRck (30.0 mg, 0.0412 mmol), and DMF (200 μL). The mixture was stirred for 3 h and the solvent removed in vacuo. The crude product was dried under vacuum to remove DMF and then purified by column chloromatography (5−8% MeOH/ CH_2Cl_2) to give **4** (35 mg, 64% yield) as a white solid. Anal. Calcd for $C_{78}H_{86}ClF_3N_7O_{20}$: C%, 61.07; H%, 5.65; N%, 6.39. Found: C%, 60.69; H%, 5.61; N%, 6.20. MS calcd for $C_{76}H_{87}ClN_7O_{19}$, 1435.5667; found, 1436.5756 $[M + H]^+$.

Synthesis of C2′-TBS-C7-succinyl-paclitaxel-phe-phe-arg chloromethyl ketone (8). To a solution of C2′-TBS-C7-succinyl paclitaxel **7** (94 mg) in 2.5 mL of CH_2Cl_2 was added EEDQ (48 mg). After stirring at room temperature for 30 min, Et_3N (40 μL) was added. The reaction mixture was transferred to a suspension of FFRck (94 mg) in 0.4 mL of DMF and allowed to stir overnight. The reaction mixture was concentrated under vacuum and the residue purified by column chromatography (3−8% MeOH/CH_2Cl_2) to give C2′-TBS-7-succinyl FFRck paclitaxel **8** (84 mg, 56%) as a white solid. The product was used in the next step without further characterization.

Synthesis of 7-succinyl PTX-phe-phe-arg chloromethyl ketone C7-PTX-FFRck (9). C2′-TBS-7-succinyl-paclitaxel-FFRck, **8** (84 mg) was treated with 2 mL of 5% HCl in MeOH. The reaction was monitored by LC-MS. After completion of reaction, the mixture was diluted with CH_2Cl_2 and quenched with $NaHCO_3$ (sat. aq.). The aqueous layer was separated and extracted with CH_2Cl_2 and the combined organic phase washed with brine, dried over sodium sulfate, and concentrated in vacuo. The residue was purified by column chromatography (8% MeOH/DCM) to give C7-PTX-FFRck, **9** (61 mg, 78%) as a white solid. Anal. Calcd for $C_{78}H_{86}ClF_3N_7O_{20}$: C%, 61.07; H%, 5.65; N%, 6.39. Found: C%, 59.71; H%, 5.93; N%, 6.41. MS calcd for $C_{76}H_{87}ClN_7O_{19}$, 1435.5667; found, 1436.5746 $[M + H]^+$.

Cell Culture. Human breast cancer cells MDA-MB-231 were obtained from American Type Cell Collection (ATCC: Rockville, MD). KB 3-1 (paclitaxel-sensitive, parental line of KB V1) and KB V1 (paclitaxel resistant) cells were the kind gifts of Dr. Michael M. Gottesman, the NCI.[34] D-MEM/F12 (a 1:1 Dulbecco's modified Eagle's medium: Ham's F12) medium, D-MEM/high glucose medicum, and penicillin G/streptomycin and L-glutamine were purchased from Mediatech (Herndon, VA), Hyclone (Logan, UT), and GIBCO-BRL/Invitrogen (Rockville, MD), respectively. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA).

KB 3-1 cells were maintained in D-MEM/F12 and KB-V1 cells were maintained in D-MEM/F12 containing vinblastine (1 μg/mL). MDA-MB-231 cells were maintained in D-MEM/high glucose. All media contained 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and 2 mM L-glutamine. Cells were incubated at 37 °C in 5% CO_2, 95% air in a humidified atmosphere.

Neutral Red (NR) Dye Cell Viability Assay for Testing Efficacy of Drugs. The efficacies of C2′-PTX-FFRck, C7-PTX-FFRck, PTX-FFRmk-fVIIa, and paclitaxel were determined with the cell viability assay in triplicate employing NR dye uptake by surviving cells as previously described.[33] The NR dye is taken up only by viable cells. Briefly, cells were plated into a 96-well plate at 20000 cells/200 μL/well, incubated, and allowed to adhere for 24 h. Then, drugs were added to appropriate wells in various concentrations in triplicate. Subsequently, cells were incubated for 72 h. At the termination of culture, medium was removed and 200 μL of fresh, warm medium containing 50 μg of NR/mL was added to each well in a 96-well plate. Cells were incubated at 37 °C for 30 min, followed by two washes with 200 μL of PBS. The NR taken up by cells was dissolved by adding 200 μL of 0.5 N HCl containing 35% ethanol. Then, the amount of the dye in each well was read at 570 nm by a Universal Microplate Reader (EL800, Bio-Tek, Winooski, VT). Results from triplicate samples were recorded as optical density units (OD_{570}) and averaged after subtraction of the blank.

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Supporting Information Available: Activities of PTX, C2′-PTX-FFRck, and C2′-PTX-FFRmk-fVIIa against PTX-sensitive (human head and neck cancer cells KB 3-1 and TU212) and PTX-resistant (KB-V1) cells) and ^{1}H NMR of compounds **4** (C2′-PTX-FFRck) and **9** (C7-PTX-FFRck). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kumar, N. Taxol-Induced Polymerization of Purified Tubulin. *J. Biol. Chem.* **1981**, *256*, 10435–10441. (b) Horwitz, S. B. Mechanism of action of taxol. *Trends Pharmacol. Sci.* **1992**, *13*, 134–136.

(2) Rooseboom, M.; Commandeur, J. N.; Vermeulen, N. P. Enzyme-catalyzed activation of anticancer prodrugs. *Pharmacol. Rev.* **2004**, *56*, 53–102.

(3) Abraham, S.; Guo, F.; Li, L. S.; Rader, C.; Liu, C.; Barbas, C. F.; Lerner, R. A.; Sinha, S. C. Synthesis of the next-generation therapeutic antibodies that combine cell targeting and antibody-catalyzed prodrug activation. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 5584–5589.

(4) Michaud, L. B.; Valero, V.; Hortobagyi, G. Risks and Benefits of Taxanes in Breast and Ovarian Cancer. *Drug Safety* **2000**, *23*, 401–428.

(5) Ojima, I.; Geng, X.; Wu, X.; Qu, C.; Borella, C. P.; Xie, H.; Wilhelm, S. D.; Leece, B. A.; Bartle, L. M.; Goldmacher, V. S.; Chari, R. V. Tumor-Specific Novel Taxoid−Monoclonal Antibody Conjugates. *J. Med. Chem.* **2002**, *45*, 5620–5623.

(6) Guillemard, V.; Saragovi, H. U. Taxane-antibody conjugates afford potent cytotoxicity, enhanced solubility, and tumor target selectivity. *Cancer Res.* **2001**, *61*, 694–699.
(7) Jaime, J.; Page, M. Paclitaxel immunoconjugate for the specific treatment of ovarian cancer in vitro. *Anticancer Res.* **2001**, *21*, 1119–1128.
(8) Banzato, A.; Bobisse, S.; Rondina, M.; Renier, D.; Bettella, F.; Esposito, G.; Quintieri, L.; Melendez-A, L.; Mazzi, U.; Zanovello, P.; Rosato, A. A. Paclitaxel−Hyaluronan Bioconjugate Targeting Ovarian Cancer Affords a Potent In vivo Therapeutic Activity. *Clin. Cancer Res.* **2008**, *14*, 3598–3606.
(9) Zhao, H.; Wang, J.-C.; Sun, Q.-S.; Luo, C.-L.; Zhang, Q. RGD-based strategies for improving antitumor activity of paclitaxel-loaded liposomes in nude mice xenografted with human ovarian cancer. *J. Drug Targeting* **2009**, *17*, 10–18.
(10) Lin, Y.; Tungpradit, R.; Sinchaikul, S.; An, F.; Liu, D.; Phutrakul, S.; Chen, S. Targeting the Delivery of Glycan-Based Paclitaxel Prodrugs to Cancer Cells via Glucose Transporters. *J. Med. Chem.* **2008**, *51*, 7428–7441.
(11) Liong, M.; Lu, J.; Kovochich, M.; Xia, T.; Ruehm, S. G.; Nel, A. E.; Tamanoi, F.; Zink, J. I. Multifunctional Inorganic Nanoparticles for Imaging, Targeting, and Drug Delivery. *ACS Nano* **2008**, *2*, 889–896.
(12) Huang, X.; El-Sayed, I. H.; Qian, W.; El-Sayed, M. A. Cancer Cell Imaging and Photothermal Therapy in the Near-Infrared Region by Using Gold Nanorods. *J. Am. Chem. Soc.* **2006**, *128*, 2115–2120.
(13) Chen, J.; Wang, D.; Xi, J.; Au, L.; Siekkinen, A.; Warsen, A.; Li, Z.; Zhang, H.; Xia, Y.; Li, X. Immuno Gold Nanocages with Tailored Optical Properties for Targeted Photothermal Destruction of Cancer Cells. *Nano Lett.* **2007**, *7*, 1318–1322.
(14) Gobin, A. M.; Lee, M.; Halas, N.; James, W. D.; Drezek, R. A.; West, J. L. Near-Infrared Resonant Nanoshells for Combined Optical Imaging and Photothermal Cancer Therapy. *Nano Lett.* **2007**, *7*, 1929–1934.
(15) Folkman, J. Angiogenesis: an organizing principle for drug discovery. *Nat. Rev. Drug Discovery* **2007**, *6*, 273–286.
(16) Zhang, Y.; Deng, Y.; Wendt, T.; Liliensiek, B.; Bierhaus, A.; Greten, J.; He, W.; Chen, B.; Hach-Wunderle, V.; Waldherr, R.; Ziegler, R.; Mannel, D.; Stern, D. M.; Nawroth, P. P. Intravenous somatic gene transfer with antisense tissue factor restores blood flow by reducing tumor necrosis factor expression and fibrin deposition in mouse meth-A sarcoma. *J. Clin. Invest.* **1996**, *97*, 2213–2224.
(17) Huang, X.; Molema, G.; King, S.; Watkins, L.; Edington, T. S.; Thorpe, P. E. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science* **1997**, *275*, 547–550.
(18) Hu, Z.; Sun, Y.; Garen, A. Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8161–8166.
(19) Nemerson, Y. Tissue factor and hemostasis. *Blood* **1988**, *71*, 1–8.
(20) Williams, E. B.; Krishnaswamy, S.; Mann, K. G. Zymogen/enzyme discrimination using peptide chloromethyl ketones. *J. Biol. Chem.* **1989**, *264*, 7536–7545.
(21) Banner, D. W.; D'Arcy, A.; Chene, C.; Winkler, F. K.; Guha, A.; Konigsberg, W. H.; Nemerson, Y.; Kirchhofer, D. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* **1996**, *380*, 41–46.
(22) Sorensen, B. B.; Persson, E.; Freskgard, P.-O.; Kjalke, M.; Ezban, M.; Williams, T.; Rao, L.V. M. Incorporation of an active site inhibitor in factor VIIa alters the affinity for tissue factor. *J. Biol. Chem.* **1997**, *272*, 11863–11868.
(23) (a) Schoellmann, G.; Shaw, E. Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochemistry* **1963**, *2*, 252–255. (b) Kettner, C.; Shaw, E. Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone. *Methods Enzymol.* **1981**, *80*, 826–842.
(24) Sun, A.; Shoji, M.; Lu, Y. J.; Liotta, D. C.; Snyder, J. P. Synthesis of EF24-tripeptide chloromethyl ketone: a novel curcumin-related anticancer drug delivery system. *J. Med. Chem.* **2006**, *49*, 3153–3158.
(25) Shoji, M.; Sun, A.; Kisiel, W.; Lu, Y.; Shim, H.; Mccarey, B. E.; Nichols, C.; Parker, E.; Pohl, J.; Mosley, C. A.; Alizadeh, A. R.; Liotta, D. C.; Snyder, J. P. Targeting tissue factor-expressing tumor angiogenesis and tumors with EF24 conjugated to factor VIIa. *J. Drug Targeting* **2008**, *16*, 185–197.
(26) Deutsch, H. M.; Glinski, J. A.; Hernandez, M.; Haugwitqt, R. D.; Narayanan, V. L.; Suffness, M.; Zalkow., L. H. Synthesis of Congeners and Prodrugs; Water-Soluble Prodrugs of Taxol with Potent Antitumor Activity. *J. Med. Chem.* **1989**, *32*, 788–792.
(27) Zakharian, T. Y.; Seryshev, A.; Balaji Sitharaman, B.; Gilbert, B. E. A Fullerene−Paclitaxel Chemotherapeutic: Synthesis, Characterization, and Study of Biological Activity in Tissue Culture. *J. Am. Chem. Soc.* **2005**, *127*, 12508–12509.
(28) Turunen, B. J.; Ge, H.; Oyetunji, J.; Desino, K. E.; Vasandani, V.; Güthe, S.; Himes, R. H.; Audus, K. L.; Seelig, A.; Georg, G. I. Paclitaxel succinate analogs: anionic and amide introduction as a strategy to impart blood−brain barrier permeability. *Bio. Med. Chem. Lett.* **2008**, *18*, 5971–5974.
(29) Liu, C.; Strobl, J. S.; Bane, S.; Schilling, J. K.; McCracken, M.; Chatterjee, S. K.; Rahim-Bata, R.; Kingston, D. G. I. Design, Synthesis, and Bioactivities of Steroid-Linked Taxol Analogues as Potential Targeted Drugs for Prostate and Breast Cancer. *J. Nat. Prod.* **2004**, *67*, 152–159.
(30) Mellado, W.; Magri, N. F.; Kingston, D. G. I.; Garcia-Arenas, R.; Orr, G. A.; Horwitz, S. B. Preparation and biological activity of taxol acetates. *Biochem. Biophys. Res. Commun.* **1984**, *124*, 329–336.
(31) Magri, N. F.; Kingston, D. G. I. Modified taxols, 4. Synthesis and biological activity of taxols modified in the side chain. *J. Nat. Prod.* **1988**, *51*, 298–306.
(32) Kant, J.; Huang, S.; Wong, H.; Fairchild, C.; Vyas, D.; Farina, V. Studies toward structure−activity relationships of Taxol. Synthesis and cytotoxicity of Taxol. Analogues with C-2′ modified phenylisoserine side chains. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2471–2474.
(33) (a) Zhang, S. Z.; Lipsky, M. M.; Trump, B. F.; Hsu, I. C. Neutral Red (NR) Assay for Cell Viability and Xenobiotic-Induced Cytotoxicity in Primary Cultures of Human and Rat Hepatocytes. *Cell Biol. Toxicol.* **1990**, *6*, 219–234. (b) Ciapetti, G.; Granchi, D.; Verri, E.; Savarino, L.; Cavedagna, D.; Pizzoferrato, A. Application of a combination of neutral red and amido black staining for rapid, reliable cytotoxicity testing of biomaterials. *Biomaterials* **1996**, *17*, 1259–1261.
(34) Akiyama, S.; Fojo, A.; Hanover, J. A.; Pastan , I.; Gottesman, M. M. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs . *Som. Cell. Mol. Genet.* **1985**, *11* (2), 117–126.