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Modulating paclitaxel bioavailability for targeting prostate cancer

Srinivas K. Kumar, Simon A. Williams, John T. Isaacs, Samuel R. Denmeade and Saeed R. Khan*

The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins School of Medicine, 1650 Orleans Street, Baltimore, MD 21231, USA

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Abstract—Four novel water-soluble peptide-paclitaxel conjugates were designed and synthesized as prostate-specific antigen (PSA)-activated prodrugs for prostate cancer therapy. These prodrugs were composed of a peptide, HSSKLQ or SSKYQ, each of which is selectively cleavable by PSA; a self-immolative linker, either para-aminobenzyl alcohol (PABS) or ethylene diamine (EDA); and the parent drug, paclitaxel. Introduction of a PABA or EDA linker between the peptide and paclitaxel in prodrugs **2**–**5** resulted in products with an increased rate of hydrolysis by PSA. The stability of prodrugs **2** and **3**, with the PABA linker, was poor in the serum-containing medium because of the weak carbonate bond between the PABA and paclitaxel; however, this disadvantage was overcome by introducing a carbamate bond using an EDA linker in prodrugs **4** and **5**. Thus, the incorporation of an EDA linker increased both the stability and PSA-mediated activation of these prodrugs. The cytotoxicity of each prodrug, as compared to paclitaxel, was determined against a variety of cell lines, including the PSA-secreting CWR22Rv1 prostate cancer cell line. The EDA-derived prodrug of paclitaxel **5** was stable and capable of being efficiently converted to an active drug that killed cells specifically in the presence of PSA, suggesting that this prodrug and similarly designed PSA-cleavable prodrugs may have potential as prostate cancer-specific therapeutic agents.

1. Introduction

Prostate cancer is the second most fatal cancer in American men. At present, there is no effective therapy for men with metastatic prostate cancer. Androgen ablation, although of substantial palliative benefit, is ultimately not curative, and the disease progresses to an androgen-independent state. While accumulating clinical data have suggested that newer chemotherapeutic agents may prolong survival in a subset of men, the dose and duration of administration of these drugs are often limited by their significant systemic toxicities. Thus, it would be highly desirable to develop site-directed anti-prostate cancer prodrugs. We therefore began to develop such agents that can be locally activated by the serine protease prostate-specific antigen (PSA).[1,2] Although serum PSA levels can exceed 1000 ng/mL in men with prostate cancer, PSA is enzymatically inactivated by binding to the major protease inhibitors α1-antichymotrypsin and α2-macroglobulin, which are at a 104- to 105-fold molar excess. In the immediate extra-tumoral environment, however, PSA retains its activity, which allows for selective prostate prodrug activation. Denmeade and coworkers have previously identified a peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) that is selectively and efficiently hydrolyzed by PSA.[3,4] We have subsequently developed a doxorubicin prodrug that incorporates this HSSKLQ peptide and has demonstrated that the prodrug can be selectively activated by PSA both in vitro and in vivo.[1,2]

Paclitaxel is an anticancer drug that demonstrates efficacy in a variety of human tumors, including prostate cancer.[5a,b] In particular, taxanes such as paclitaxel have been reported to have significant clinical activity in

Abbreviations: DIEPA, *N*,*N*′-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; EDA, ethylenediamine; EDC, N-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; ESI-MS, electrospray ionization-mass spectrometry; Fmoc, *N*-(9-fluorenylmethoxycarbonyl); HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HPLC, high pressure liquid chromatography; LC–MS, liquid chromatography–fed mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; Mu, morpholinocarbonyl; NMR, nuclear magnetic resonance; PSA, prostate-specific antigen; PABA, *p*-aminobenzylalcohol; RP-HPLC, reversed-phase high pressure liquid chromatography; SF, serum-free.

* Corresponding author. Tel.: +1 410 614 0200; fax: +1 410 614 8397; e-mail: khansa@jhmi.edu

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hormone-refractory prostate cancer.[16] However, paclitaxel's lack of selectivity and aqueous solubility presents serious drawbacks with regard to its use in conventional cancer chemotherapy. Its reported side effects include neutropenia, mucositis, cardiac and neurological toxicities, and hypersensitivity.

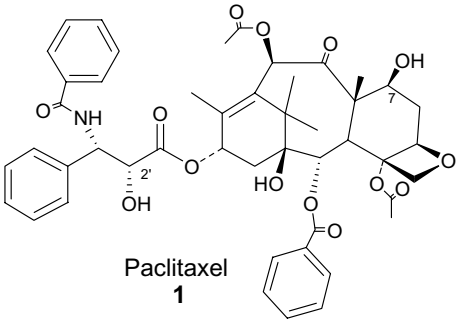
While extensive work has been done to develop paclitaxel prodrugs,[6] most efforts have been focused on obtaining derivatives with improved water solubility,[7–13] and only a few attempts have been made to convert paclitaxel into prodrugs with both enhanced water solubility and tumor-recognizing properties.[14,15] In an attempt to achieve these goals, we have begun to develop water-soluble paclitaxel prodrugs that can only be activated when processed proteolytically by the PSA present within the prostate cancer metastasis itself. By applying the successful strategy that we previously used for the 5-fluorodeoxyuridine prodrug,[17] we have now developed a series of four paclitaxel prodrugs **2**–**5** by coupling paclitaxel to the selectively PSA-cleavable peptide Mu-HSSKLQ (where Mu = morpholinocarbonyl protecting group) or MuSSKYQ, via a self-cleaving diamino acid linker,[18] either para-aminobenzylalcohol (PABA) or ethylenediamine (EDA) (Chart 1).

2. Results and discussion

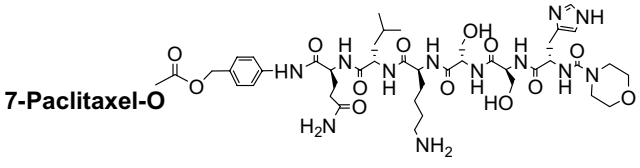
2.1. Chemistry

SAR studies have shown that while modification of the 2′-hydroxyl group of paclitaxel results in a loss of activity, the 2′-hydroxyl group is the most convenient site for attaching designed functional domains.[19–21] 2′-MuHSSKLQ-derived paclitaxel prodrugs would ideally have low cytotoxicity and more stability in serum, while releasing active drug at the tumor site in a PSA-dependent manner. We have previously synthesized three 2′-paclitaxel prodrugs with diamino linkers: 2′-MuHSSKLQFP-paclitaxel, 2′-MuHSSKLQMeLP-paclitaxel, and 2′-MuHSSKLQ-LAib-paclitaxel, respectively. We also synthesized a prodrug by attaching the peptide HSSKLQLAib to paclitaxel at the 7-hydroxyl position (unpublished data).

Prodrugs with PABA linkers **2** and **3** were synthesized as depicted in Schemes 1 and 2. On treatment of paclitaxel with *N*-benzyloxycarbonylsuccinamide, 2′-OH of the paclitaxel was protected to give compound **6**. Compound **6**, on reacting with trichloromethylchloroformate (diphosgene) in the presence of triethylamine, gave 7-chloroformatepaclitaxel **7**. Fmoc-Gln-PABA **10** was

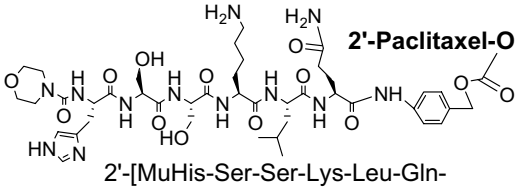


Paclitaxel
1

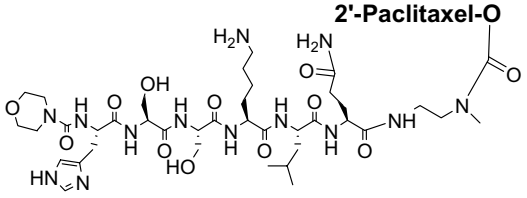


7-[MuHis-Ser-Ser-Lys-Leu-Gln-PABC]Paclitaxel

2

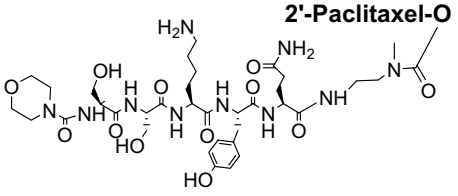


2'-[MuHis-Ser-Ser-Lys-Leu-Gln-PABC]Paclitaxel **3**



2'-[MuHis-Ser-Ser-Lys-Leu-Gln-NH-$(CH_2)_2$-$N(CH_3)$]-Paclitaxel

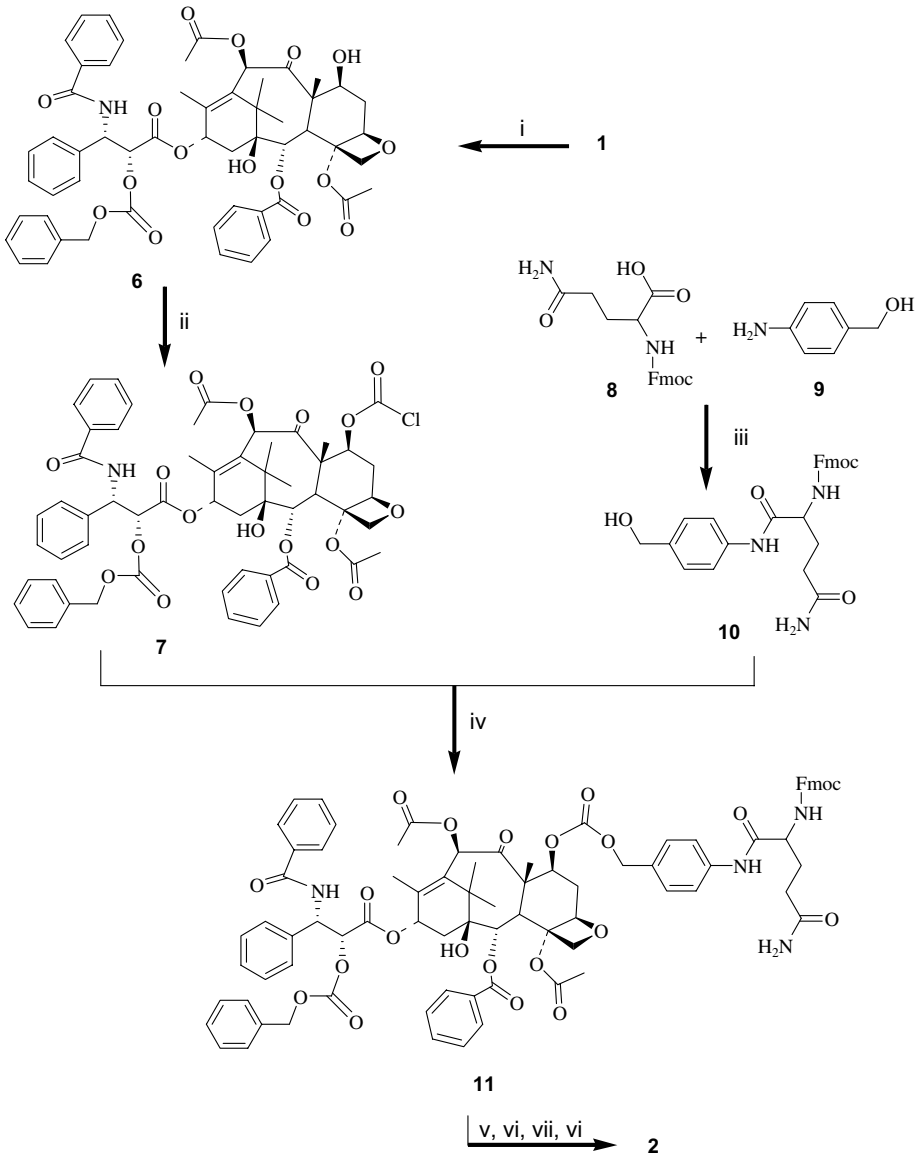
4



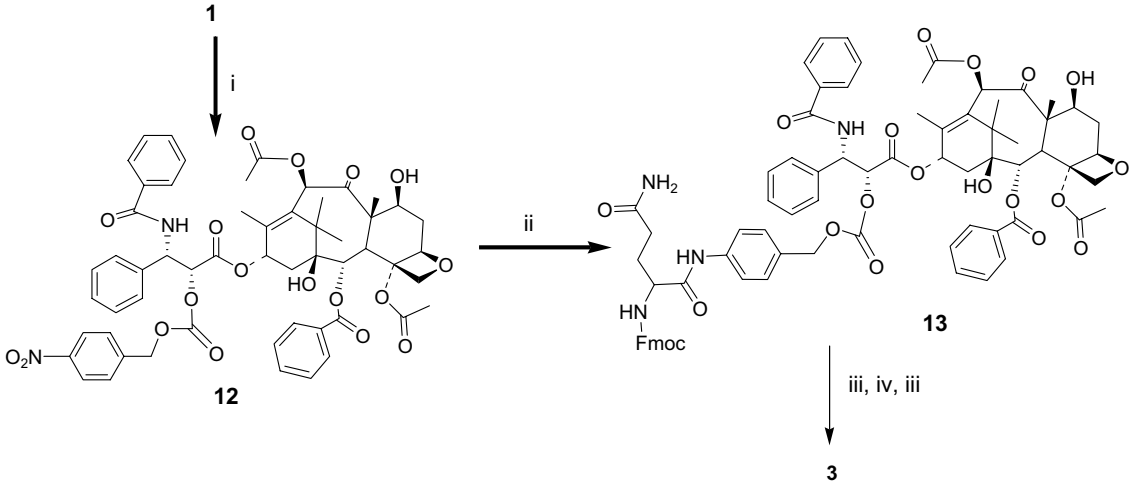
2'-[Mu-Ser-Ser-Lys-Tyr-Gln--NH-$(CH_2)_2$-$N(CH_3)$]-Paclitaxel

5

Chart 1.



Scheme 1. Reagents and conditions: (i) *N*-(benzyloxycarbonyloxy)succinamide; (ii) diphosgene, triethylamine; (iii) isobutylchloroformate, DIPEA; (iv) DIPEA, DCM; (v) H_2/Pd, 4 h; (vi) 20% Piperidine/DMF; (vii) MuHSSK(Fmoc)L-OH, HBTU, DIPEA/DMF.



Scheme 2. Reagents and conditions: (i) *p*-nitrochloroformate, DIPA; (ii) **10**, DIPA; (iii) 20% piperidine/DMF; (iv) MuHSSK(Fmoc)L-OH, EDC, DIPA/DMF.

prepared by coupling Fmoc-Gln-OH **8** with para-aminobenzyl alcohol (**9**) in the presence of isobutylchloroformate. Chloroformatepaclitaxel **7**, on stirring with **10** in the presence of DIPEA, gave 7-Fmoc-Gln-PABC-(2′-Z-Paclitaxel) **11**. Deprotection of Z-group at the 2′ position of **11** with H_2/Pd and coupling with the peptide MuHSSK(Fmoc)L in the presence of HBTU, followed by the deprotection of Fmoc with 20% piperidine, gave prodrug **2** (Scheme 1). Similarly, prodrug **3** was prepared starting with **12**, which was obtained by coupling paclitaxel with *p*-nitrophenyl chloroformate. Compound **12**, on reacting with **10** in the presence of DIPEA, gave **13**. Deprotection of Fmoc on **13** and coupling with the peptide MuHSSK(Fmoc)L in the presence of EDC, followed by the deprotection of Fmoc with 20% piperidine, gave prodrug **3** (Scheme 2).

Prodrug **4**, with EDA as the linker, was synthesized starting with Z-Gln-OH **14** (Scheme 3). Compound **4** was coupled with the Boc-protected EDA **15** to give **16**. Compound **16**, on reacting with compound **12** in the presence of DIPEA, gave **17**. Deprotection of Z group on compound **17** and coupling with the peptide MuHSSK(Fmoc)L in the presence of EDC, followed by the deprotection of Fmoc with 20% piperidine, gave prodrug **4**. A similar procedure was followed for the synthesis of prodrug **5** using the peptide MuSSK(Fmoc)Y-OH. All the above intermediates and the final product were characterized by ESI-MS, MALDI, ^{1}H NMR, and HPLC.

The synthesized paclitaxel prodrugs were readily water-soluble (i.e., up to 70 mg/mL, ~38 μmol/mL), with much improved water solubility when compared to that of paclitaxel (0.00025 mg/mL, ~0.2 μmol/mL).[22]

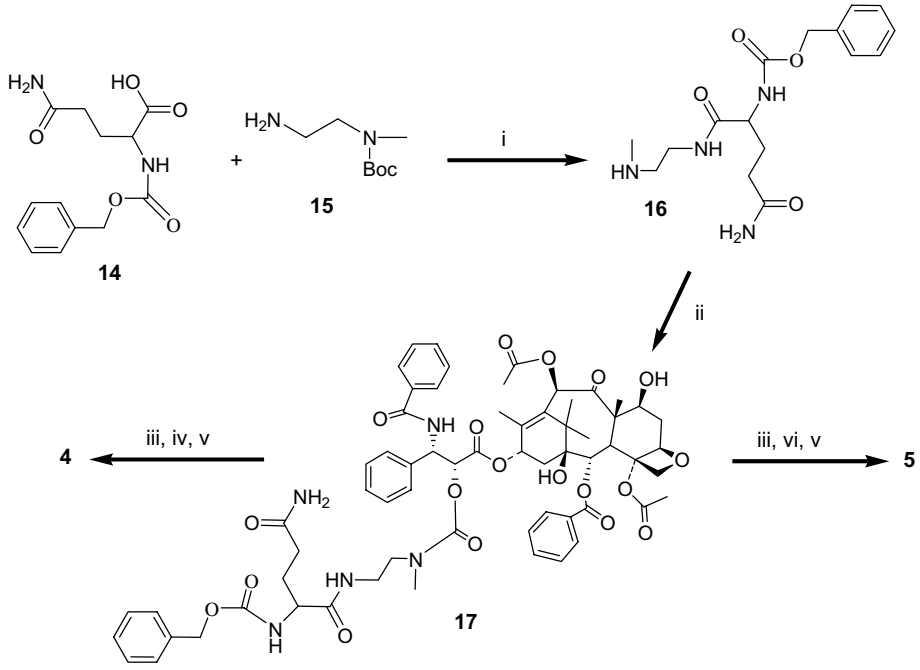
2.2. Biology

2.2.1. Stability of the prodrugs in buffer solution and serum-free medium. For stability testing, prodrugs **2**–**5** at a concentration of 100 μM were individually stored in 0.1 M Tris/HCl buffer (pH 7.8) for 48 h at 37 °C. Stability was then assessed by HPLC, injecting 100 μL of each incubated solution. The data showed that no parent drug was formed from any of the four prodrugs, and all four prodrugs were stable in the buffer solution for 48 h at 37 °C. Similar results were found when the same four prodrugs were incubated in serum-free medium.

2.2.2. Stability of the prodrugs in plasma and serum. Prodrugs **2**–**5** at 100 μM were individually incubated in plasma for 48 h at 37 °C. Cold acetonitrile (500 μL) was added at various times during the incubation (at 2, 4, 8, 16 32, 40, and 48 h) to 500-μL aliquots of each prodrug-plasma mixture and then centrifuged. The supernatants were injected onto the HPLC column, and the stability of the four prodrugs was monitored. A similar procedure was followed to evaluate the stability of the same prodrugs in serum. In plasma, 7% and 10% of compounds **2** and **3**, respectively, were found to be degraded over a period of 48 h, whereas compounds **4** and **5** showed less than 3% degradation in plasma. However, incubation of compounds **2** and **3** with serum showed 92% and 87% degradation, respectively, over a period of 48 h, whereas under the same conditions compounds **4** and **5** showed less than 2% degradation.

3. Discussion

The synthesized paclitaxel prodrugs were readily water-soluble (i.e., up to 70 mg/mL, ~38 μmol/mL), with much



Scheme 3. Reagents and conditions: (i) isobutylchloroformate, DIPA; (ii) **12**, DIPA; (iii) H_2/Pd, 4 h; (iv) MuHSSK(Fmoc)L-OH,EDC, DIPA, DMF; (v) 20% Piperidine/DMF; (vi) MuSSK(Fmoc)Y-OH,EDC, DIPA, DMF.

Table 1. Half-lives of the compounds **2**–**5** in various media

Compound	Half-lives of the paclitaxel prodrugs **2**–**5** in various media[a,b]				
	+PSA[c]	−PSA[c]	Plasma	Serum	Media
2′-(MuHSSKLQ-PABC)-Paclitaxel (**2**)	14 h	NH	NH	1.5 h	NH
7-MuHSSKLQ-PABC-Paclitaxel (**3**)	12 h	NH	NH	1 h	NH
2′-(MuHSSKLQ-EDA)-Paclitaxel (**4**)	10 h	NH	NH	NH	NH
2′-(MuSSKYQ-EDA)-Paclitaxel (**5**)	6 h	NH	NH	NH	NH

NH: not hydrolyzed significantly.
[a] Half-lives represent an average of triplicates.
[b] Initial prodrug concentration: 100 μM.
[c] Buffer solution (50 mM Tris and 0.1 M NaCl, pH 7.8).

improved water solubility when compared to paclitaxel (0.00025 mg/mL, ~0.2 μmol/mL).[22]

The prodrugs **2**–**5** were incubated with PSA in PBS buffer solution (pH 7.8) in order to assess their ability to be hydrolyzed by the PSA enzyme to yield peptide (MuHSSKLQ- or MuSSKYQ-) and release free paclitaxel. The reactions were monitored by RP-HPLC and LC-MS (ESI). After incubation with PSA in PBS, prodrugs **2** and **3** showed a 50% conversion (i.e., half-life) to the peptide MuHSSKLQ and free paclitaxel over a period of 14 h for **2** and 12 h for **3** (Table 1). This rate represented a significant increase in the rate of hydrolysis of these two prodrugs when compared to the previously developed diamino acid-linked paclitaxel prodrugs (unpublished data). For both **2** and **3**, the intermediate cleavage product PABC-paclitaxel was not detected, demonstrating instantaneous cyclization of the linker and release of paclitaxel after the cleavage of the peptide by PSA.

Compound **2** with the PABA linker showed a better rate of hydrolysis after incubation with PSA than did compound **3**, and therefore it was chosen for further enzyme-substrate kinetics studies. In vitro, PSA cleaved paclitaxel from prodrug **2** with a $K_m = 216.45$ μM, $K_{cat} = 0.00585\ s^{-1}$, and $K_{cat}/K_m = 27.03\ M^{-1}\ s^{-1}$. These kinetics were similar to those previously reported for the fluorescent substrate MuHSSKLQ//AMC (K_m = 470 μM, $K_{cat} = 0.011\ s^{-1}$, $K_{cat}/K_m = 23.6\ M^{-1}\ s^{-1}$)[3].

In initial studies, the in vitro IC_{50} (concentration required for 50% growth inhibition) values were determined for prodrugs **2**–**3** against non-PSA-secreting TSU cells by treating the cells for 48 h with various concentrations of the prodrugs in the presence and absence of exogenous PSA. Paclitaxel prodrugs **2** and **3** showed reduced cytotoxicity against the TSU cell line when compared to the parent drug paclitaxel (i.e., 30% vs 90% growth inhibition at 1 nM concentration, respectively (Figs. 1 and 2). The addition of active PSA to the medium of these TSU cells slightly enhanced the cytotoxicity of both **2** and **3**. Further stability testing of these prodrugs for stability in a variety of media demonstrated that while both **2** and **3** were completely stable to hydrolysis in human plasma and buffer, both prodrugs were readily hydrolyzed in human serum. Since the cytotoxicity assays require the presence of serum for cell viability, these results suggest that non-specific hydrolysis of the prodrugs by active proteases in the serum results in significant activation of the prodrug in the absence of active PSA.

To overcome this problem, we synthesized compounds **4** and **5** containing the EDA linker. The PSA hydrolysis rates for these compounds were indeed improved when

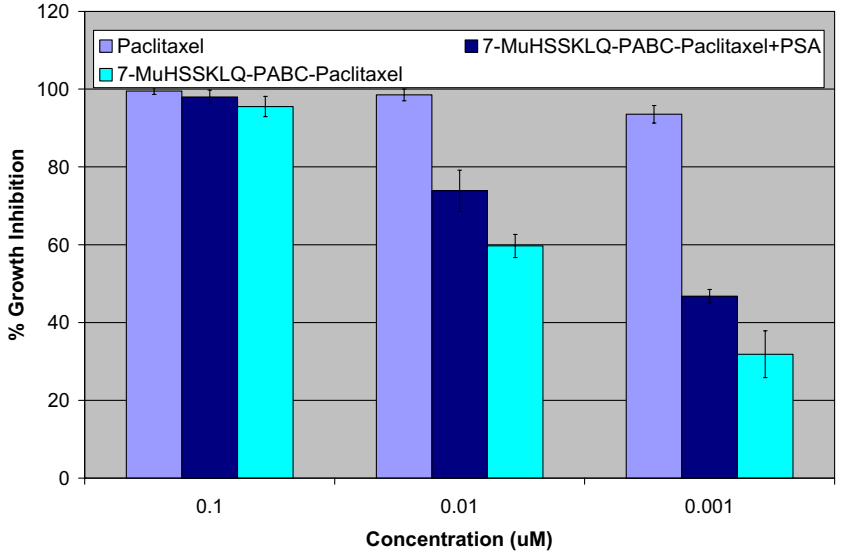


Figure 1. Effect of the 7-MuHSSKLQ-PABC-paclitaxel prodrug (**2**) ± PSA on TSU cells.

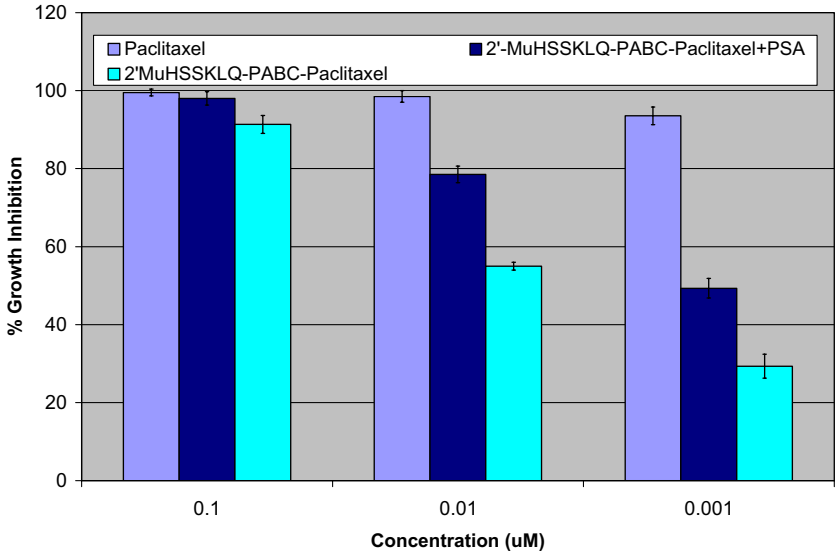


Figure 2. Effect of the 2′-MuHSSKLQ-PABC-paclitaxel (**3**) prodrug ± PSA on TSU cells.

compared to those for the prodrugs with the PABA linker. The half-life (i.e., 50% hydrolysis point) in PSA was 10 h for compound **4** and 6 h for compound **5** (Table 1). This result was comparable to those in previous studies using fluorescent substrates that had indicated that the peptide used in compound **5**, Mu-SSKYQ, was a better substrate for PSA (unpublished data).

Stability studies demonstrated that, in contrast to compounds **2** and **3** (Figs. 3 and 4), compounds **4** and **5** with the EDA linker were completely resistant to non-specific hydrolysis in both human plasma and serum (Figs. 5 and 6). Based on these stability studies and the hydrolysis profiles of prodrugs **2**–**5** (Table 1), we tested the activity of the 2′-(MuSSKYQ)-paclitaxel prodrug (**5**) against human prostate cancer cell lines CWR22Rv1 (PSA-producing) and DU145 (PSA non-producing), and the human bladder cancer cell line TSU (PSA non-producing). Each of these cell lines was highly sensitive to paclitaxel with IC_{50} for growth inhibition of <1 nM (data not shown). In contrast, the peptide moiety Mu-SSKYQ lacking the attached paclitaxel demonstrated no toxicity to these cell lines at concentrations up to 100 μM (data not shown). After 96 h of treatment, the CWR22Rv1 cell line showed a 3- to 5-fold higher level of cytotoxicity (IC_{50} of approximately 1 μM) to the 2′-(MuSSKYQ)-paclitaxel prodrug than did the non-PSA-secreting cell lines Du145 and TSU (Fig. 7). This result corroborates the results of our initial experiments in which we added exogenous PSA to cells that do not secrete endogenous protease.

4. Conclusions

In summary, we have synthesized four novel water-soluble paclitaxel prodrugs. Hydrolysis of these prodrugs with PSA indicated that using EDA linker between the PSA-cleavable peptide and the paclitaxel increased both the stability of the prodrug in serum and its recognition

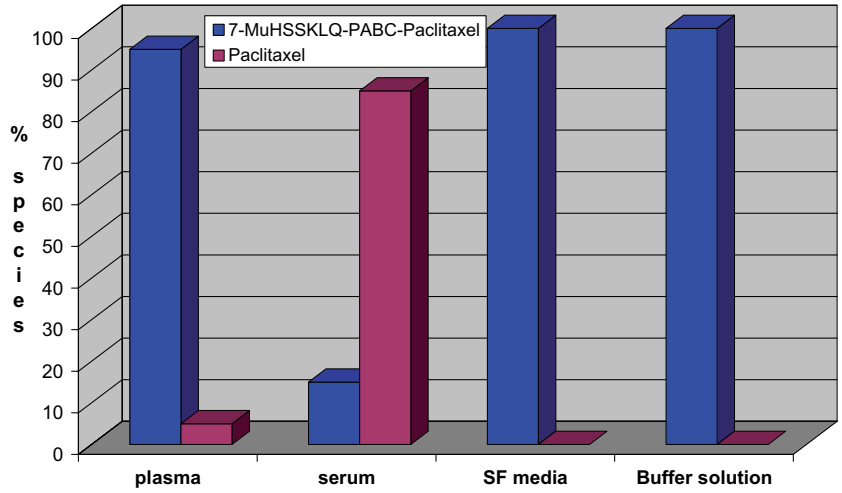


Figure 3. Stability of the 7-MuHSSKLQ-PABC-paclitaxel (**2**) prodrug in various media at 37 °C after 48 h. SF, serum-free medium.

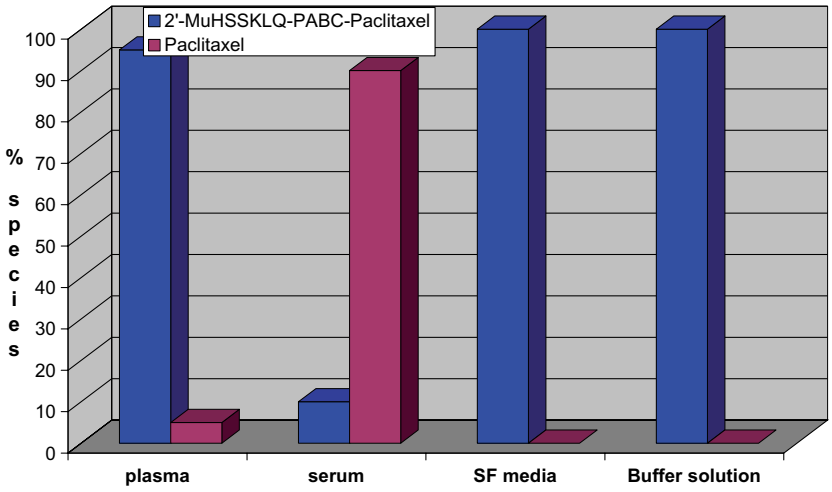


Figure 4. Stability of the 2′-MuHSSKLQ-PABC-paclitaxel (**3**) prodrug in various media at 37 °C after 48 h.

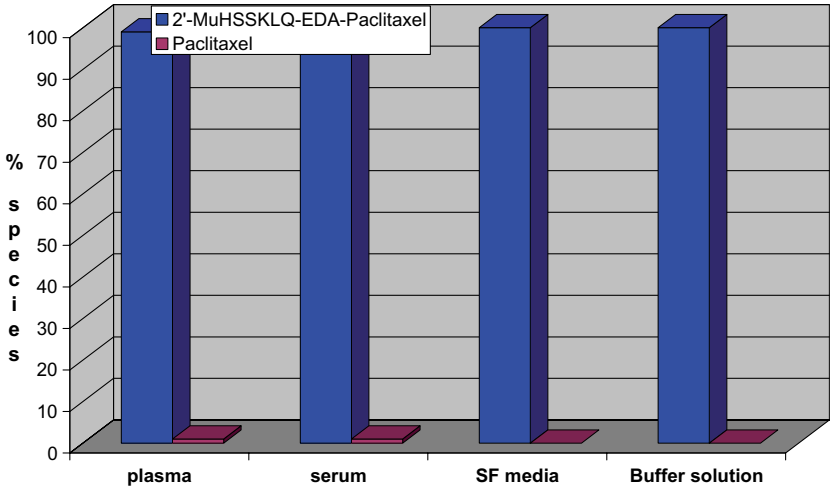


Figure 5. Stability of the 2′-MuHSSKLQ-EDA-paclitaxel (**4**) prodrug in various media at 37 °C after 48 h.

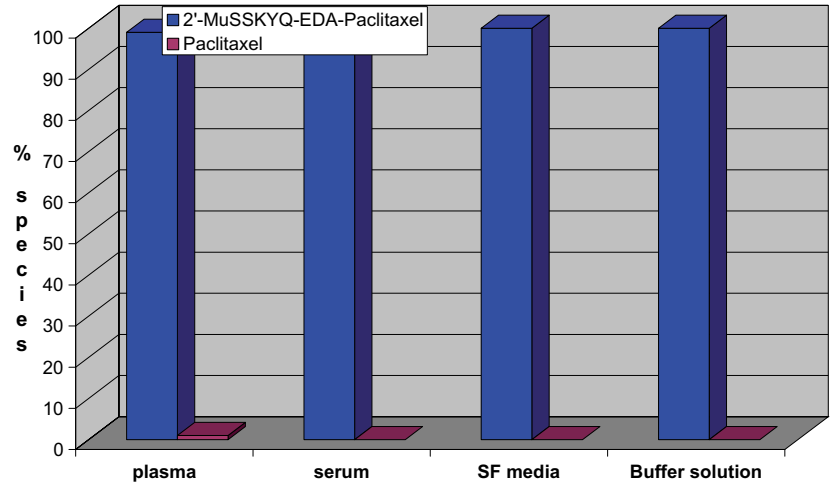


Figure 6. Stability of the 2′-MuSSKYQ-EDA-paclitaxel (**5**) prodrug in various media at 37 °C after 48 h.

and cleavage by PSA. Although prodrugs **2** and **3** were unstable in medium containing serum, they both showed instantaneous release of free paclitaxel when incubated with PSA in the buffer solution. Cytotoxicity assays using the prodrugs indicated that the 2-hydroxyl-derived prodrug of paclitaxel **5** was quite stable and could be

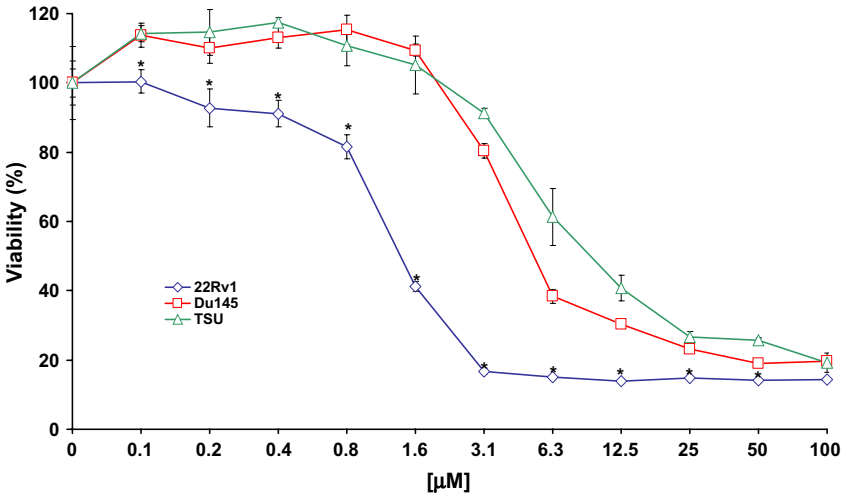


Figure 7. The 2′-(MuSSKYQ-EDA)-paclitaxel prodrug (**5**) selectively inhibits the viability of PSA-expressing prostate cancer cell lines. PSA-secreting CWR22Rv1 cells and PSA-null Du145 and TSU cells were treated for 96 h with prodrug at various doses in medium with 1% serum before being tested in MTT viability assays. Results were normalized to the viability of cells in the absence of prodrug. Data shown are from a representative experiment, with viability assays performed in replicates of 8. $^{*}P < 0.05$.

efficiently converted to an active drug that killed prostate cancer cells in the presence of PSA. This EDA linker-derived paclitaxel prodrug with peptide MuSSKYQ showed potent PSA-selective anti-tumor capability in vitro when incubated with prostate cancer cells. The results obtained with these agents point to the usefulness of further studies to characterize the potential of these prodrugs, particularly prodrug **5**, as prostate cancer-specific therapeutic agents.

5. Experimental

5.1. General methods

All reactions were carried out in a nitrogen atmosphere under anhydrous conditions, unless otherwise noted. All reagents and solvents were purchased from Sigma–Aldrich, USA, and used without further purification. NMR spectra were recorded on a 400-MHz Varian NMR spectrometer. Chemical shifts are reported as ppm (δ units) relative to tetramethylsilane as an internal standard. The purity of the prodrugs was checked with RP-HPLC. All compounds were at least 95% pure. HPLC analyses were carried out on a Waters system equipped with photodiode array detector and either a 150×4.60 mm 5μ C18 (analytical runs) or a 250×10.00 mm 10μ C18 (preparative runs) reversed-phase column. Mass spectra were obtained with a Voyager DE-STR (Applied Biosystems) MALDI-TOF or Bruker LC-MS mass spectrometer. Flash column chromatography was performed with Merck Kieselegel 60, and TLC analysis was performed on Analtech precoated silica gel 60 GF-254 plates.

5.2. 2′-*Z*-Paclitaxel (6)

N-Benzyloxycarbonyloxysuccinamide (290 mg, 1.17 mmol, 5 eq.) in 10 mL THF was added to a solution of paclitaxel (200 mg, 0.23 mmol) and triethylamine (236 mg, 2.3 mmol, 10 eq.) in THF (6 mL) at room temperature and allowed to stir for 16 h. The solvent was removed under vacuum, and the residue was purified by column chromatography to yield 160 mg of compound **6** as white powder (68%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (6H, s), 1.28 (3H, s), 1.72 (5H, m), 2.00 (2H, m), 2.02 (3H, s), 2.05 (3H, s), 2.2 (1H, OH), 2.78 (1H, s), 3.38 (2H, m), 4.00 (1H, s), 4. 5 (1H, m), 4.94 (2H, dd), 5.52 (1H, s), 5.44 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 7.0 (1H, s), 7.12 (2H,), 7.19 (5H, m), 7.24 (2H,), 7.37 (2H, d), 7.46 (3H, m), 7.51 (1H, m), 7.99 (2H, m), 8.0 (1H, NH); MS (EI) *m/e* 989 $(M+H)^+$, 1010 $(M+Na)^+$. Anal. ($C_{55}H_{57}NO_{16}$) C, H, N.

5.3. 2′-*Z*-7-(Chloroformate)-paclitaxel (7)

A solution of 100 mg (0.1 mmol) of 2′-*Z*-paclitaxel (**6**) and *N*,*N*′-diisopropylethylamine (26 mg, 2 eq) in methylene chloride (3 mL) at room temperature under argon was treated with 10 mg of trichloromethylchloroformate (0.05 mmol). After 4 h, the crude product was evaporated to dryness in vacuo to give 90 mg of 2′-*Z*-7-(chloroformate)-paclitaxel (**7**) (84%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (6H, s), 1.28 (3H, s), 1.72 (3H, s), 1.82 (2H, d), 2.00 (2H, m), 2.02 (3H, s), 2.05 (3H, s), 2.2 (1H, OH), 2.78 (1H, s), 3.38 (1H, m), 4.00 (1H, s), 4.12 (1H, t), 4.5 (1H, m), 4.94 (2H, dd), 5.52 (1H, s), 5.44 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 7.0 (1H, s), 7.12 (2H,), 7.19 (5H, m), 7.24 (2H,), 7.37 (2H, d), 7.45 (3H, m), 7.51 (1H, m), 7.99 (2H, m), 8.0 (1H, NH); MS (EI) *m/e* 1051 $(M+H)^+$, 1072 $(M+Na)^+$. Anal. ($C_{56}H_{56}ClNO_{17}$) C, H, N.

5.4. Fmoc-Gln-PABA (10)

A solution of 500 mg (1.36 mmol) Fmoc-Gln-OH (**8**) was dissolved in dry tetrahydrofuran (6 mL) under an argon atmosphere and cooled to −20 °C. One hundred and thirty-seven milligrams of 4-methylmorpholine (1.36 mmol) and 185 mg (1.36 mmol) of

isobutylchloroformate were added. The reaction mixture was stirred for 4 h at −20 °C. A solution of 167 mg (1.36 mmol) 4-aminobenzyl alcohol in dry tetrahydrofuran (4 mL) was added dropwise, and the reaction mixture was allowed to come to room temperature and then stirred for 16 h. Tetrahydrofuran was evaporated, and dichloromethane was added. The organic layer was washed with a saturated sodium bicarbonate solution, a 0.5 N potassium bisulfate solution, and brine, then dried over anhydrous sodium sulfate and evaporated. The residual crude product was purified by column chromatography (ethyl acetate–hexane, 8:2) to yield 59 mg of the Fmoc-Gln-PABA **10** (92%): ^{1}H NMR (400 MHz, CD_3OD) δ 2.17 (2H, m), 2.18 (2H, m), 4.4 (1H, m), 4.5 (1H, m), 4.79 (2H, dd), 7.17 (2H, m), 7.2 (2H, m), 7.4 (2H, m), 7.57 (4H, m), 7.84 (2H, d),; MS (EI) *m/e* 474 $(M+H)^+$, 495 $(M+Na)^+$. Anal. ($C_{27}H_{27}N_3O_5 \cdot H_2O$) C, H, N.

5.5. 2′-*Z* -7-(Fmoc-Gln-PABA)-paclitaxel (11)

To a solution of 80 mg (0.07 mmol) **7** in dry tetrahydrofuran-dichloromethane (2 mL, 1:1) under an argon atmosphere were added 44 mg (0.09 mmol, 1.2 eq) **10** and *N*,*N*′-diisopropylethylamine (20 mg, 2 eq). The reaction mixture was stirred at room temperature for 48 h. After 48 h, 25 mL of ethyl acetate was added. The organic layer was washed with 10% citric acid, brine, and water, then dried over anhydrous sodium sulfate and evaporated. The residue was subjected to column chromatography (ethyl acetate–hexane, 1:1; chloroform–methanol, 30:1, respectively) to afford 60 mg of the desired carbonate **11** (58%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (6H, s), 1.28 (3H, s), 1.72 (5H, m), 2.00 (2H, m), 2.02 (3H, s), 2.05 (3H, s), 2.17 (2H, m), 2.18 (2H, m), 2.2 (1H, OH), 2.78 (1H, s), 3.38 (2H, m), 4.00 (1H, s), 4.4 (1H, m), 4.5 (2H, m), 4.79 (2H, dd), 4.94 (2H, dd), 5.52 (1H, s), 5.44 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 7.0 (1H, s), 7.12 (2H,), 7.19 (9H, m), 7.4 (2H, m), 7.24 (2H,), 7.37 (2H, d), 7.46 (3H, m), 7.51 (5H, m), 7.84 (2H, d), 7.99 (2H, m), 8.2 (1H, NH); MS (EI) *m/e* 1488 $(M+H)^+$, 1509 $(M+Na)^+$. Anal ($C_{83}H_{82}N_4O_{22}$) C, H, N.

5.6. 7-[Mu-His-Ser-Ser-Lys-Leu-Gln-PABA]paclitaxel (2)

To a solution of 50 mg (0.033 mmol) of compound **11** in ethyl acetate was added a catalytic amount of 10% Pd-C. The mixture was shaken for 3 h under an H_2 atmosphere. The Pd-C was removed by filtering through Celite. The solvent was evaporated to give 40 mg (0.029 mmol, 89%) of 7-(Fmoc-Gln-PABA)-paclitaxel. After addition of 20% piperidine in DMF, the mixture was stirred for 30 min to give 7-(Gln-PABA)-paclitaxel, which was further dried in vacuo for 2 h and utilized for the following coupling reaction without further purification: To a cooled solution of Mu-His-Ser-Ser-Lys(Fmoc)-Leu-OH (22 mg, 0.024 mmol), HBTU (9.2 mg, 0.024 mmol), and the above 7-(Gln-PABA)-paclitaxel in 0.5 mL DMF, DIEPA (8 μL, 0.046 mmol) were added and stirred for 6 h. To this mixture, acetonitrile and water (1:1) (10 mL) was added, and the product was purified by HPLC to give 25 mg (0.015 mmol, 65%) of compound **2**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.0 (6H, m, 2 CH_3-Leu), 1.21 (6H, s), 1.27 (3H, s), 1.29 (2H, m, CH_2-Lys), 1.54 (2H, m, CH_2-Lys), 1.72 (3H, m), 1.75 (2H, m, CH_2-Leu), 1.80 (3H, m, CH_2-Lys and CH-Leu), 1.9 (2H, m), 2.00 (2H, m), 2.02 (3H, s), 2.05 (3H, s), 2.16-2.18 (8H, m), 2.58 (2H, m, CH_2-Leu), 2.78 (1H, s), 3.05 (2H, m, CH_2-His), 3.38 (2H, m), 3.47 (4H, m, CH_2-morpholine), 3.67 (4H, m, CH_2-morpholine), 4.0 (2H, m, Hα), 4.4 (2H, m), 4.5 (2H, m), 4.6 (3H, m, Hα), 4.65 (4H, m, $2CH_2$-Ser), 4.79 (2H, dd), 4.85 (1H, m, Hα), 4.94 (2H, dd), 5.52 (1H, s), 5.44 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 6.8 (1H, CH-His), 7.0 (1H, s), 7.12 (2H,), 7.15 (2H, d), 7.19 (9H, m), 7.24 (2H,), 7.37 (2H, d), 7.4 (2H, m), 7.44 (1H, CH-His), 7.46 (3H, m), 7.52 (7H, m), 7.84 (2H, d), 8.0 (2H, m). MS (MALDI) *m/e* 1797 $(M+H)^+$, 1818 $(M+Na)^+$. Anal ($C_{89}H_{113}N_{13}O_{27} \cdot H_2O$) C, H, N.

5.7. 2′-[4-Nitrophenyl-carbonate]paclitaxel (12)

To a solution of 200 mg (0.23 mmol) paclitaxel in dry dichloromethane under an argon atmosphere was added pyridine (4 drops). At −50 °C, 275 mg (6.0 eq) of 4-nitrophenyl chloroformate dissolved in dry dichloromethane was added. The reaction mixture was stirred at −50 °C, and after 4 h, 4-nitrophenyl chloroformate (4.2 eq) was added. After 1 h, the mixture was diluted with dichloromethane, washed with 0.5 N potassium bisulfate and brine, and dried over anhydrous sodium sulfate. After evaporation of the solvents, the residual yellow film was purified by means of column chromatography (ethyl acetate–hexane, 1:1) to yield 125 mg of activated paclitaxel **7** (65%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17 (s, 3H), 1.24 (s, 3H), 1.69 (s, 3H), 1.92 (s, 3H), 2.25 (s, 3H), 2.49 (s, 3H), 2.55 (m, 1H), 3.82 (d, 1H), 4.24 (d, 1H), 4.38 (d, 1H), 4.42 (m, 1H), 4.96 (bd, 1H), 5.53 (d, 1H), 5.70 (d, 1H), 6.10 (dd, 1H), 6.29 (s, 1H), 6.34 (m, 1H), 6.90 (d, 1H), 7.34 (d, 2H), 7.37–7.65 (m, 11H), 7.75 (d, 2H), 8.15 (d, 2H), 8.26 (d, 2H); MS (FAB) *m/e* 1020 $(M+H)^+$, 1042 $(M+Na)^+$. Anal. ($C_{54}H_{54}N_2O_{18}$) C, H, N.

5.8. 2′-Fmoc-Gln-PABA-paclitaxel (13)

To a solution of 100 mg (0.09 mmol) **12** in dry tetrahydrofuran-dichloromethane (5 mL, 1:1) under an argon atmosphere were added 48 mg (0.09 mmol) **10** and *N*,*N*′-diisopropylethylamine (25 mg, 2 equiv). The reaction mixture was stirred at room temperature for 48 h. After 48 h, ethyl acetate was added. The organic layer was washed with 10% citric acid, brine, and water, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by means of column chromatography (ethyl acetate–hexane, 1:1; chloroform–methanol, 30:1, respectively) to afford 106 mg of the desired carbonate **13** (79%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (6H, s), 1.29 (3H, s), 1.75 (5H, m), 2.0 (2H, m), 2.12 (3H, s), 2.15 (3H, s), 2.17 (4H, m), 2.2 (1H, OH), 2.78 (1H, s), 3.38 (2H, m), 4.00 (1H, s), 4.4 (1H, m), 4.5 (2H, m), 4.79 (2H, dd), 4.94 (2H, dd), 5.42 (1H, s), 5.7 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 7.0 (1H, s), 7.12 (2H,), 7.19 (4H, m), 7.4 (2H, m), 7.24 (2H,), 7.37 (2H,

d), 7.46 (3H, m), 7.51 (5H, m), 7.84 (2H, d), 7.99 (2H, m), 8.2 (1H, NH); MS (EI) *m/e* 1354 $(M+H)^+$, 1375 $(M+Na)^+$. Anal ($C_{75}H_{76}N_4O_{20}$) C, H, N.

5.9. 2′-[Mu-His-Ser-Ser-Lys-Leu-Gln-PABA]paclitaxel (3)

To a solution of 50 mg (0.03 mmol) of compound **13** was added 20% piperidine in DMF (3 mL). After the mixture was stirred for 30 min, the solvent was removed in vacuo and recrystallized in hexane to give 38 mg (92%) of 2′-(Gln-PABA)-paclitaxel, which was further dried in vacuo for 2 h and utilized for the following coupling reaction without further purification: To a solution of Mu-His-Ser-Ser-Lys(Fmoc)-Leu-OH (22 mg, 0.024 mmol), 4.6 mg (0.024 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and 27 mg (0.024 mmol) of the above 2′-(Gln-PABA)-paclitaxel in 0.5 mL DMF, *N*,*N*′-diisopropylethylamine (6 mg, 2 eq) was added and stirred for 6 h. To this mixture, acetonitrile and water (10 mL, 1:1) were added and the product was purified by HPLC to yield 22 mg of **3** (52%): ^{1}H NMR (400 MHz, $CDCl_3$) δ^1H NMR (400 MHz, $CDCl_3$) δ 1.0 (6H, m, $2CH_3$-Leu), 1.21 (6H, s), 1.27 (3H, s), 1.29 (2H, m, CH_2-Lys), 1.54 (2H, m, CH_2-Lys), 1.72 (5H, m), 1.75 (2H, m, CH_2-Leu), 1.80 (3H, m, CH_2-Lys and CH-Leu), 2.00 (2H, m), 2.02 (3H, s), 2.05 (3H, s), 2.16–2.18 (8H, m), 2.58 (2H, m, CH_2-Leu), 2.78 (1H, s), 3.03 (2H, m, CH_2-His), 3.4 (2H, m), 3.47 (4H, m, CH_2-morpholine), 3.67 (4H, m, CH_2-morpholine), 4.1 (2H, m, Hα), 4.4 (2H, m), 4.5 (2H, m), 4.6 (3H, m, Hα), 4.65 (4H, m, $2CH_2$-Ser), 4.79 (2H, dd), 4.85 (1H, m, Hα), 4.94 (2H, dd), 5.5 (1H, s), 5.44 (2H, dd), 5.80 (1H, d), 6.10 (1H, d), 6.8 (1H, CH-His), 7.0 (1H, s), 7.12 (2H,), 7.15 (2H, d), 7.19 (9H, m), 7.24 (2H,), 7.37 (2H, d), 7.4 (2H, m), 7.44 (1H, CH-His), 7.46 (3H, m), 7.52 (7H, m), 7.84 (2H, d), 8.0 (2H, m). MS (MALDI) *m/e* 1797 $(M+H)^+$, 1818 $(M+Na)^+$. Anal ($C_{89}H_{113}N_{13}O_{27}\cdot 2H_2O$) C, H, N.

5.10. Z-Gln-NH-$(CH)_2$-NH(Me) (16)

To a suspension of 1 g (3.57 mmol) Z-Gln-OH (**14**) in dry tetrahydrofuran (25 mL) under an argon atmosphere at −50 °C were added 4-methylmorpholine (0.58 mL, 5.35 mmol) and 0.485 g (3.57 mmol) isobutylchloroformate. After 2 h, this mixture was added dropwise to a solution of 0.685 g (3.92 mmol) monoprotected *N*-methylethylenediamine (**15**) in dry dichloromethane at −50 °C. After 90 min, the solvent was evaporated; water and dichloromethane were added to the resulting solid. The residue obtained by filtration was subjected to column chromatography (chloroform–methanol–acetic acid, 95:3:2), yielding 1.48 g of the desired product Z-Gln-NH-$(CH)_2$-N(Me)-Boc (**16**) (90%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (9H, s), 1.98 (2H, m), 2.18 (2H, t), 2.81(2H, m), 3.32 (2H, m), 4.53 (1H, m), 2.47 (3H, d), 5.33 (2H, dd), 6.5 (2H, NH_2), 7.2 (5H, m), 5.0 (1H, NH); MS (EI) *m/e* 438 $(M+H)^+$, 459 $(M+Na)^+$.

One gram (2.28 mmol) of **Z-Gln-NH-$(CH)_2$-N(Me)-Boc** was dissolved in ethyl acetate (15 mL), and 30 mL of 4 M hydrochloric acid was added. After 4 h, the mixture was concentrated in vacuo, and 20 mL of *tert*-butyl alcohol was added to the residual product and evaporated. The product was freeze-dried in dioxane to obtain 770 mg of the desired product **16** (99%), which was used without further purification. A small portion of **16** was recrystallized in ethyl acetate–methanol. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.07 (2H, m), 2.18 (2H, t), 2.81(2H, m), 3.0 (1H, NH), 3.32 (2H, m), 4.53 (1H, m), 2.47 (3H, d), 5.34 (2H, dd), 6.1 (2H, NH_2), 7.19 (5H, m), 8.0 (1H, NH); MS (EI) *m/e* 337 $(M+H)^+$, 358 $(M+Na)^+$. Anal ($C_{16}H_{24}N_4O_4$) C, H, N.

5.11. Z-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel (17)

To a solution of 500 mg (0.49 mmol) of the activated paclitaxel **12** in dry DMF (3 mL) under an argon atmosphere was added 4-methylmorpholine (0.1 mL, 2 eq). At −30 °C, a solution of 165 mg (0.49 mmol) **16** (1.0 eq) and 4-methylmorpholine (0.05 mL, 1 eq) dissolved in dry DMF (2 mL) was added dropwise. The reaction mixture was stirred at −20 °C for 2.5 h. The solution was diluted with 10% 2-propanol-ethyl acetate (25 mL) and washed with sodium bicarbonate, 0.5 N potassium bisulfate, brine, and water. The organic layer was dried (sodium sulfate) and concentrated in vacuo. The residue was subjected to column chromatography (ethyl acetate–hexane, 9:1) to give 356 mg of compound **17** (60%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (6H, s), 1.28 (3H, s), 1.72 (5H, m), 2.00 (2H, m), 2.02 (3H, s), 2.05 (5H, s), 2.2 (2H, t), 2.47 (3H, d), 2.80 (3H, m), 3.0 (1H, NH), 3.32 (2H, m), 3.38 (2H, m), 4.00 (1H, s), 4. 5 (2H, m), 4.94 (2H, dd), 5.34 (2H, dd), 5.44 (2H, dd), 5.52 (1H, s), 5.80 (1H, d), 6.10 (3H, m), 7.0 (1H, s), 7.12 (2H, m), 7.19 (10H, m), 7.24 (2H,), 7.37 (2H, d), 7.46 (3H, m), 7.51 (1H, m), 7.99 (2H, m), 8.0 (1H, NH); MS (MALDI) *m/e* 1217 $(M+H)^+$, 1238 $(M+Na)^+$. Anal. ($C_{64}H_{73}N_5O_{19}$) C, H, N.

5.12. Mu-His-Ser-Ser-Lys-Leu-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel (4)

To a solution of 50 mg (0.04 mmol) of protected Z-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel (**17**) in 5% acetic acid–methanol was added a catalytic amount of 10% Pd-C. The mixture was stirred for 3 h under an H_2 atmosphere. The Pd-C was removed by centrifugation, using Celite as the filtering agent. Ethyl acetate was added, and the solvent was evaporated in vacuo. *tert*-Butyl alcohol was added and removed by evaporation in vacuo, and the residue was freeze-dried to yield 42 mg (96%) of the deprotected product **Gln-NH-$(CH)_2$-NH(Me)-paclitaxel**: ^{1}H NMR (400 MHz, CD_3OD) δ 1.21 (6H, s), 1.26 (3H, s), 1.72 (5H, m), 2.00 (2H, m), 2.02 (3H, s), 2.05 (5H, s), 2.2 (2H, t), 2.47 (3H, d), 2.80 (3H, m), 3.0 (1H, NH), 3.32 (2H, m), 3.36 (2H, m), 4.03 (1H, s), 4. 52 (2H, m), 4.92 (2H, dd), 5.44 (2H, dd), 5.52 (1H, s), 5.80 (1H, d), 6.10 (3H, m), 7.0 (1H, s), 7.12 (2H, m), 7.15 (5H, m), 7.26 (2H,), 7.38 (2H, d), 7.45 (3H, m), 7.6 (1H, m), 7.99 (2H, m), 8.0 (1H, NH); MS (FAB) *m/e* 1083 $(M+H)^+$, 1104 $(M+Na)^+$. Anal. ($C_{57}H_{65}N_5O_{17}\cdot 2H_2O$) C, H, N.

Diisopropylethylamine (8 μL, 0.046 mmol) was added to a solution of 22 mg (0.024 mmol) of Mu-His-Ser-Ser-

Lys(Fmoc)-Leu-OH, 4.6 mg (0.024 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and 26 mg (0.024 mmol) of the above deprotected Gln-NH-$(CH)_2$-NH(Me)-paclitaxel in DMF (0.5 mL) and stirred for 6 h. To this mixture, acetonitrile and water (1:1) (10 mL) were added, and the product was purified by HPLC to obtain the desired product: 32 mg (68%) of Mu-His-Ser-Ser-Lys(Fmoc)-Leu-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel. Deprotection of the Fmoc on the Mu-His-Ser-Ser-Lys(Fmoc)-Leu-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel was carried out by stirring with 20% piperidine in DMF for 30 min. To this product, acetonitrile and water (1:1) (10 mL) were added, and the resulting product was purified by HPLC to give 22 mg (80%) of the desired Mu-His-Ser-Ser-Lys-Leu-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel ^{1}H NMR (400 MHz, CD_3OD) δ 1.0 (6H, m, $2CH_3$-Leu), 1.21 (6H, s), 1.26 (3H, s), 1.29 (2H, m, CH_2-Lys), 1.54 (2H, m, CH_2-Lys), 1.71 (5H, m),1.76 (2H, m, CH_2-Leu), 1.80 (3H, m, CH_2-Lys and CH-Leu), 2.00 (2H, m), 2.02 (3H, s), 2.05 (5H, s), 2.18 (4H, m, CH_2-Gln), 2.2 (2H, t), 2.47 (3H, d), 2.58 (2H, m, CH_2-Leu), 2.80 (3H, m), 3.0 (1H, NH), 3.05 (2H, m, CH_2-His), 3.32 (2H, m), 3.36 (2H, m), 4.03 (1H, s), 3.49 (4H, m, CH_2-morpholine), 3.63 (4H, m, CH_2-morpholine), 4.1 (2H, m, Hα), 4.5 (3H, m, Hα), 4. 52 (2H, m), 4.6 (4H, m, 2 CH_2-Ser), 4.92 (1H, m, Hα), 4.96 (2H, dd), 5.44 (2H, dd), 5.5 (1H, s), 5.81 (1H, d), 6.12 (3H, m), 6.82 (1H, CH-His), 7.0 (1H, s), 7.12 (2H,), 7.15 (5H, m), 7.26 (2H, m), 7.38 (2H, d), 7.44 (1H, CH-His), 7.45 (3H, m), 7.6 (1H, m), 8.1 (2H, m). MS (MALDI) *m/e* 1748 $(M+H)^+$, 1769 $(M+Na)^+$. Anal. ($C_{85}H_{114}N_{14}O_{26}$) C, H, N.

5.13. Mu-Ser-Ser-Lys-Tyr-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel (5)

Diisopropylethylamine (8 μL, 0.046 mmol) was added to a solution of 20 mg (0.024 mmol) of Mu-Ser-Ser-Lys(Fmoc)-Tyr-OH, 4.6 mg (0.024 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and 26 mg (0.024 mmol) of the deprotected Gln-NH-$(CH)_2$-NH(Me)-paclitaxel in DMF (0.5 mL) and stirred for 6 h. To this mixture, acetonitrile, and water (1:1) (10 mL) were added, and the product was purified by HPLC to obtain the desired product: 35 mg (80%) of Mu-Ser-Ser-Lys(Fmoc)-Tyr-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel. Deprotection of the Fmoc on the Mu-Ser-Ser-Lys(Fmoc)-Try-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel was carried out by stirring with 20% piperidine in DMF for 30 min. To this product, acetonitrile, and water (1:1) (10 mL) were added, and the resulting product was purified by HPLC to give 23 mg (60%) of the desired Mu-Ser-Ser-Lys-Try-Gln-NH-$(CH)_2$-NH(Me)-paclitaxel (**5**). ^{1}H NMR (400 MHz, CD_3OD) δ 1.0 (6H, m, $2CH_3$-Leu), 1.21 (6H, s), 1.26 (3H, s), 1.29 (2H, m, CH_2-Lys), 1.54 (2H, m, CH_2-Lys), 1.71 (5H, m),1.76 (2H, m, CH_2-Leu), 1.80 (3H, m, CH_2-Lys and CH-Leu), 2.00 (2H, m), 2.02 (3H, s), 2.05 (5H, s), 2.18 (4H, m, CH_2-Gln), 2.2 (2H, t), 2.47 (3H, d), 2.58 (2H, m, CH_2-Leu), 2.80 (3H, m), 3.0 (1H, NH), 3.05 (2H, m, CH_2-His), 3.32 (2H, m), 3.36 (2H, m), 4.03 (1H, s), 3.49 (4H, m, CH_2-morpholine), 3.63 (4H, m, CH_2-morpholine), 4.1 (2H, m, Hα), 4.5 (3H, m, Hα), 4. 52 (2H, m), 4.6 (4H, m, 2 CH_2-Ser), 4.92 (1H, m, Hα), 4.96 (2H, dd), 5.44 (2H, dd), 5.5 (1H, s), 5.81 (1H, d), 6.12 (3H, m), 6.82 (1H, CH-His), 7.0 (1H, s), 7.12 (2H,), 7.15 (5H, m), 7.26 (2H, m), 7.38 (2H, d), 7.44 (1H, CH-His), 7.45 (3H, m), 7.6 (1H, m), 8.1 (2H, m). MS (MALDI) *m/e* 1661 $(M+H)^+$, 1682 $(M+Na)^+$. Anal. ($C_{82}H_{105}N_{11}O_{26}$) calcd C 59.30, H 6.37, N; found C 58.88, H 5.92, N.

5.14. Kinetics of enzymatic hydrolysis

Various concentrations of 7-[Mu-HSSKLQ-PABA]paclitaxel (**2**) were incubated with PSA (10 μg/ mL final concentration) in 50 mM Tris and 0.1 M NaCl (pH 7.8) at room temperature. At discrete time points (0.5, 1, 2, 3, 4, 8, and 14 h), aliquots of the reaction mixture were removed and analyzed by HPLC on a reversed-phase C18 Phenomenex analytical column (25 cm × 4.6 mm). A standard curve produced by using purified free paclitaxel was used to convert peak area to free paclitaxel concentrations. Peak areas of free paclitaxel at each time point were then converted to concentration, and the concentration data were analyzed by Lineweaver–Burk plots ($1/V$ vs $1/S$, where V = reaction velocity and S = substrate concentration). K_m, V_{max}, and K_{cat} were calculated from these plots, and the ratio of K_{cat} to K_m was derived.

5.15. In vitro cytotoxicity

The antiproliferative effect of prodrugs **2**–**5** and paclitaxel was determined in vitro. The human prostate cancer cell lines CWR22Rv1 (PSA-secreting, ATCC) and Du145 (non-PSA-secreting, ATCC), and the TSU bladder cancer cell line (non-PSA-secreting, ATCC) were transferred to 96-well plates at a concentration of 2000 cells per well in RPMI 1640 with 10% serum, supplemented with 1% penicillin/streptomycin and 2 mM L-glutamine (Gibco BRL). Two days later, the medium was replaced with the desired prodrug or paclitaxel in RPMI with 1% serum for 96 h. After 96 h, MTT Dye Solution (Promega) was added to the wells and incubated for 3 h at 37 °C before the addition of solubilization/stop solution (Promega) and an overnight incubation at 37 °C. Color changes in the plate were read at an absorbance of 570 nm and normalized to the value for untreated cells. The TSU cell line was treated with exogenous PSA at a concentration of 10 μg/mL (a higher concentration of PSA than in serum [ng/mL] but lower than extra-tumoral PSA levels [mg/mL]) for 48 h in combination with the prodrug or paclitaxel.

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