The Synthesis of a Prodrug of Doxorubicin Designed to Provide Reduced Systemic Toxicity and Greater Target Efficacy

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Doxorubicin (Dox) can provide some stabilization in prostate cancer; however, its use is limited because of systemic toxicities, primarily cardiotoxicity and immunosuppression. The administration of a prodrug of doxorubicin, designed to permit selective activation by the tumor, would reduce general systemic exposure to the active drug and would thereby increase the therapeutic index. Prostate specific antigen (PSA) is a serine protease with chymotrypsin-like activity that is a member of the kallikrein gene family. PSA's putative physiological role is the liquefaction of semen by virtue of its ability to cleave the seminal fluid proteins semenogelins I and II. Serum PSA levels have been found to correlate well with the number of malignant prostate cells. The use of a prodrug which is cleaved by the enzyme PSA in the prostate should in principle produce high localized concentrations of the cytotoxic agent at the tumor site while limiting systemic exposure to the active drug. Cleavage maps following PSA treatment of human semenogelin were constructed. Systematic modification of the amino acid residues flanking the primary cleavage site led to the synthesis of a series of short peptides which were efficiently hydrolyzed by PSA. Subsequent coupling of selected peptides to doxorubicin provided a series of doxorubicin-peptide conjugates which were evaluated in vitro and in vivo as targeted prodrugs for PSA-secreting tumor cells. From these studies we selected Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox, **27**, as the peptide-doxorubicin conjugate with the best profile of physical and biological properties. Compound **27** has a greater than 20-fold selectivity against human prostate PSA-secreting LNCaP cells relative to the non-PSA-secreting DuPRO cell line. In nude mouse xenograft studies, **27** reduced PSA levels by 95% and tumor weight by 87% at a dose below its MTD. Both doxorubicin and Leu-Dox (**13**) were ineffective in reducing circulating PSA and tumor burden at their maximum tolerated doses. On the basis of these results, we selected **27** for further study to assess its ability to inhibit human prostate cancer cell growth and tumorigenesis.

Introduction

Prostate cancer is the second leading cause of cancer mortality in males. It is estimated that 37 000 men died of prostate carcinoma in the United States in 1999.[1] While cancer that is confined to the prostate can be treated with surgery or radiation, for metastatic disease the prognosis is poor. There is currently no effective therapy that significantly extends survival for men with androgen refractory prostate cancer, although palliative therapy is commonly employed.[2]

The current therapy for metastatic prostate cancer, a combination of mitoxantrone and prednisone, provides response rates of 20 to 30%.[3] Doxorubicin (**I**) has limited utility in prostate cancer due to systemic toxicities, consisting primarily of cardiotoxicity and myelosuppression.[4] The administration of a prodrug of doxorubicin, designed to permit selective activation by the tumor, would reduce general systemic exposure to the active drug and would thereby increase the therapeutic index. This strategy in turn will permit delivery of an increased amount of active drug to the target site, thereby increasing efficacy.

The concept of using a prodrug to achieve targeted delivery rather than oral bioavailability was, to our knowledge, first employed with anticancer drugs and later also with other medications. We, as well as others, have considered using the enzyme prostate specific antigen (PSA) to convert an inactive doxorubicin prodrug into the active drug at the site of the tumor.[5,6,7]

PSA is a serine protease with chymotrypsin-like activity that is a member of the kallikrein gene family.[8,9] PSA's putative physiological role is the liquefaction of semen by virtue of its ability to cleave the seminal fluid proteins semenogelins I and II.[10] Clinically PSA is best known as a serological marker for the presence of prostate cancer. Serum PSA levels have been found to correlate well with the number of malignant prostate cells, and higher levels of PSA are indicative of metastatic disease.[11] Importantly, PSA which is secreted into systemic circulation lacks enzymatic activity because it forms a complex with α_1-antichymotrypsin and α_2-macroglobulin.[12,13] This loss of enzymatic activity does

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not, however, affect the utility of complexed PSA as a serological marker for prostate cancer since the immunoreactivity is not lost. The use of a prodrug which is cleaved by the enzyme PSA should in principle produce high localized concentration of the cytotoxic agent at the tumor site while limiting systemic exposure to the active drug.

Cleavage maps following PSA treatment of human semenogelin I have been constructed by us as well as others.[14] Isolation of the cleavage fragments allowed us to determine that the most efficient cleavage occurs between Gln^{349} and Ser^{350} in semenogelin I. Systematic modification of the amino acid residues flanking this cleavage site led to the synthesis of a series of short peptides which were efficiently hydrolyzed by PSA. Subsequent coupling of selected peptides through their C-terminal carboxyl group to the amino group of doxorubicin provided a series of doxorubicin-peptide conjugates which were evaluated in vitro and in vivo as targeted prodrugs for PSA secreting tumor cells. On the basis of these studies, we selected Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox, **27**, for further study to assess its ability to inhibit human prostate cancer cell growth and tumorigenesis.

Results and Discussion

Design of Prodrug, 27. The initial steps in designing a potentially useful doxorubicin prodrug were 2-fold: first, to identify a functionality on doxorubicin where conjugation with a peptide fragment would yield a biologically poorly active compound, and second, to identify a peptide substrate that, when conjugated to doxorubicin, would be converted by PSA into the active drug, doxorubicin, in high yield. The coupling of Ac-Ala-Ala-Ala-OH to form an amide, **14**, with the aminoglycoside of doxorubicin abolished doxorubicin's cytotoxic activity on PSA secreting (LNCaP) and PSA nonsecreting (DuPRO) cells (Table 3). This observation achieved our first objective in that attachment of a peptide to the aminoglycoside of doxorubicin rendered doxorubicin inactive to these prostate cancer cell lines. The second design issue would be addressed with the discovery of an appropriate peptide substrate which would be linked to doxorubicin. As will become apparent from the discussion that follows, the selection of the peptide fragment to be coupled to doxorubicin was far from trivial.

The amino acid sequence of human semenogelin I, the physiological substrate for PSA, was determined in our laboratories (see Methods section) as well as in others.[10] Recombinant human semenogelin I was prepared and digested with a highly purified preparation of human PSA. The proteolytic digestion fragments were isolated via acrylamide gel electrophoresis and their structures determined by sequencing from the amino terminus. Five unique amino termini were identified and are indicated as CS-1 (cleavage site 1) through CS-5 (Supporting Information). The cleavage sites are numerically ranked according to the number of nanomoles of peptide produced during a 5 min cleavage reaction, with CS-1 representing the most abundant species found. Semenogelin I was shown to be primarily cleaved at the C-terminus of Gln (CS-1, CS-3) and to a lesser degree at the C-terminus of Ser and Tyr (CS-2, CS-5). There also appears to be a preference for Ser or Gln at the P1′ site (N-terminal) at all the major cleavage points. PSA's selective hydrolysis on the C-terminal side of Gln appears to be unique among known proteases. Importantly this selective Gln cleavage represents an advantage toward the development of PSA selective prodrugs in that nonspecific proteolysis would be minimized. On the basis of these considerations, a series of peptides was prepared whose sequences flanked CS-1. The peptides were tested as substrates for PSA to determine their relative efficiencies for hydrolysis by PSA (Table 1). The results are expressed as the time required for PSA to hydrolyze 50% of the peptide ($T_{1/2}$). Human semenogelin I was shown to have a $T_{1/2}$ of less than 10 min. The 25-mer, **1**, which represents P10−P15′ from CS-1 demonstrates significant cleavage with a 25 min $T_{1/2}$. Truncation to the 12-mer, **2**, increases the $T_{1/2}$ to 105 min. Further deletion to the 8-mer, **3**, and the 9-mer, **4**, dramatically increases the $T_{1/2}$ to greater than 180 and 240 min, respectively. Anticipating that ultimately blocked C and N termini would be desirable in the context of a peptide conjugate (to minimize nonspecific exopeptidase hydrolysis), the 12-mer, sequence **2**, was prepared as the *N*-acetyl C-terminal amide **5**. The $T_{1/2}$ for **5** was >240 min which represented a less effective substrate relative to the unblocked **2**. Nevertheless we chose this structure, **5**, as a starting point for the development of improved substrates.

Table 1. Synthetic Peptides and Analogues from Cleavage Site-1 (CS-1) of Semenogelin I[a]

	sequences	PSA $T_{1/2}$ (min)
	semenogelin I (1−462)	<10
1	H-Ser-Gln-Lys-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ser-Ser-Thr-Glu-Glu-Arg-Arg-Leu-His-Tyr-Gly-Glu-Asn-Gly-OH	25
2	H-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ser-Ser-Thr-Glu-OH	105
3	H-Ser-Tyr-**Gln-Ser**-Ser-Ser-Thr-Glu-OH	>180
4	H-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ser-OH	>240
5	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ser-Ser-Thr-Glu-NH_2	>240
6	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ala-Ser-Thr-Glu-NH_2	145
7	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ser-Lys-Thr-Glu-NH_2	110
8	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-**Gln-Ser**-Ala-Lys-Thr-Glu-NH_2	125
9	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-**Tyr-Ser**-Ser-Ser-Thr-Glu-NH_2	150
10	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-**Gln-Ser**-Ser-Ser-Thr-Glu-NH_2	105
11	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-**Gln-Ser**-Ala-Ser-Thr-Glu-NH_2	50

[a] PSA cleavage site between the two amino acids is indicated in bold. Underlined amino acids indicate position of substitution. $T_{1/2}$= time required for PSA to hydrolyze 50% of compound at a molar ratio of 1:100, respectively. The structure of semenogelin I is presented in the Supporting Information figure.

A series of analogues based on **5** was prepared in which libraries at P1′, P2′, P3′ and P4′ (**1L**−**4L**) were prepared by the simultaneous incorporation of an equimolar mixture of 19 common natural amino acids in their protected forms (Table 2). Synthetic details and data providing evidence for the generation of 19 component equimolar peptide libraries were presented in a previous report.[15] The four individual libraries were subjected to PSA digestion for 60 min. Following quenching, the digests were evaluated by automated Edman sequence analysis. Because only those peptides which are cleaved by PSA would give products with a free amino terminus, we were able to determine which residues were most susceptible to PSA hydrolysis.

The preferred amino acid in P1′ proved to be Ser, it was gratifying to find, the same as in the native sequence, **1L**. At P2′, Ala and Tyr were preferred over

Table 2. Preferred Substitution Based on PSA Digestion of 19 Component P1′−P4′ Peptide Libraries

	sequence[a]	preferred substitn[b]
1L	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-Gln-(19Xaa)-Ser-Ser-Thr-Glu-NH_2	P1′ = Ser
2L	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-Gln-Ser-(19Xaa)-Ser-Thr-Glu-NH_2	P2′ = Ala, Tyr
3L	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-Gln-Ser-Ser-(19Xaa)-Thr-Glu-NH_2	P3′ = Lys
4L	Ac-Ala-Asn-Lys-Ile-Ser-Tyr-Gln-Ser-Ser-Ser-(19Xaa)-Glu-NH_2	P4′ = Leu, Ile

[a] 19Xaa = equimolar mixture of each of the following amino acids: Ala,Arg,Asn,Asp,Gln,Glu,Gly,His,Ile,Leu,Lys,Met,Phe,Pro,Ser,Thr,Trp,Tyr,Val. [b] As determined by automated N-terminal sequence analysis following PSA hydrolysis.

the native Ser, **2L**. Lys at P3′ was better relative to Ser, **3L**, and at P4′ Leu and Ile were preferred over native Thr, **4L**. During the analysis of these libraries we only observed the formation of cleavage products related to the hydrolysis following Gln at P1. This observation further confirmed the selectivity of PSA for Gln. The singly prepared 12-mer, **6**, in which Ala substitutes for Ser at P2′, exhibited an improved $T_{1/2}$ of 145 min. Replacement of Ser at P3′ with Lys, **7**, reduced the $T_{1/2}$ further to 110 min. However, double substitution with Ala at P2′ and Lys at P3′, **8**, did not provide an additive effect to PSA hydrolysis with **8** showing a 125 min $T_{1/2}$. PSA hydrolysis of four peptide libraries (P1′−P4′) followed by automated Edman sequence analysis allowed us to efficiently determine the effect of point substitution relative to cleavage rates. However, a similar approach was not as readily accessible for evaluating P1−P4 substitution since PSA hydrolysis would not generate a unique set of amino terminal hydrolytic products. To determine which amino acids, within the context of the 12-mer substrate, **5**, most contributed to improved PSA cleavage, we prepared single point substitution analogues of **5** for P1 through P4 positions using the Advanced ChemTech (Louisville, KY) 396 automated multiple peptide synthesizer. The same set of 19 amino acids was introduced, using the manufacturer's Fmoc chemistry protocols, individually in P1 through P4. Following isolation from the resin, the 76 peptides, without further purification, were treated with PSA to determine their relative rates of cleavage (see Experimental Section for $T_{1/2}$ determinations). From this study we determined in a qualitative assessment that the preferred amino acids (based on $T_{1/2}$ values) at P1 are Gln, Tyr, Phe, and Asn; at P2 they are Tyr and Leu; at P3 Ser and Lys; and at P4 Ala, Ser, and Thr. Confirmation of these results was provided in part by purification of selected peptides and determination of their $T_{1/2}$. Tyr replacement at P1 provided **9**, with an improved $T_{1/2}$ of 150 min, while Ala at P4 produced a compound **10**, with a $T_{1/2}$ of 105 min (Table 1). By combining the $T_{1/2}$ enhancing properties of Ala replacement at P2′ (**8**) and Ala at P4 (**10**) a substrate, **11**, was synthesized which had a dramatically improved $T_{1/2}$ of 50 min (Table 1). As in the peptide libraries described above, only PSA cleavage products ascribed to the P1−P1′ site were observed. These results demonstrate that peptide substrates with enhanced PSA selective cleavage rates relative to a native sequence can be prepared using a systematic approach of amino acid substitution.

Synthesis of Conjugates

Peptide doxorubicin conjugates were prepared based on, in part, structural data derived from the above studies. Synthesis of the peptide-doxorubicin conjugates utilized a combination of solid-phase and solution chemistries, Scheme 1. The peptides were prepared by solid-phase synthesis using a Boc strategy on 4-(oxymethyl)-phenylacetamidomethyl (PAM) resin. The cleavage of the peptides from the resin and removal of the side chain protecting groups were carried out in anhydrous HF. Where Orn, Lys, or glutaric acid were present, Fmoc was used for side chain protection of the amino group and OFm (fluorenylmethyl ester) on the carboxyl of glutaric acid, respectively. Both groups are stable to HF. The peptides were used without purification in coupling of the C-terminal carboxyl to the doxorubicin daunosamine nitrogen using a variety of coupling agents (see Methods). Following coupling, those compounds which required removal of the Fmoc or OFm protecting group were treated with 20% piperidine in DMF at 0 °C. The peptide conjugates were purified using reverse phase high-performance liquid chromatography (RP-HPLC) with a Waters C_{18} Delta Pak (15 μm, 100 Å) column. Identity and purity of the final products were determined by amino acid analysis, HPLC, and FAB mass spectroscopy.

Biological Results

Cytotoxic activities of the peptide-doxorubicin conjugates were evaluated in cell culture. Both PSA producing, LNCaP, and PSA nonproducing, DuPRO, human prostate cancer cell lines were used for analysis. Table 3 summarizes the results of these assays. EC_{50} values report the compound dose required to kill 50% of the tumor cells. Doxorubicin (Dox) and leucyldoxorubicin (Leu-Dox), **13**, represent the active cytotoxic species generated from the peptide-doxorubicin conjugates following PSA cleavage of the Gln-Ser (P1−P1′) bond with subsequent hydrolysis within the protease rich environment of the prostate cancer cells (data not shown). PSA $T_{1/2}$ cleavage times for the conjugates are also presented in Table 3.

Leu-Dox, **13**, has been shown to be cytotoxic in a number of cancer cell lines, and its pharmacokinetics have been studied in clinical trials.[16,17] Our previous report as well as data presented herein (Table 3) show that Leu-Dox, **13**, is also cytotoxic to prostate cancer cell lines.[7] From our peptide library studies we determined that Leu was a preferred amino acid replacement at P4′ (Table 2). In light of these observations, that is, Leu-Dox's cytotoxicity to prostate cancer cells and its preference in the context of a peptide substate for PSA, we concluded that Leu is an appropriate doxorubicin linker in an extended peptide substrate. Coupling of the aminoglycoside of doxorubicin to the peptide sequence, Ac-AlaAsnLys(N^{ϵ}-Fmoc)AlaSerTyrGlnSerSerSerLeu-OH (**15a**), followed by removal of the N^{ϵ}-Fmoc group provided the conjugate **15** (Table 3). The data show that **15** has a $T_{1/2}$ of 30 min which represents a significant improvement relative to the unconjugated peptide substrates, **2**−**11** (Table 1), the best of which gave a $T_{1/2}$ of 50 min (**11**). In cell culture, selective cytotoxic activity of **15** for PSA-secreting vs non-PSA-secreting cells was demonstrated (Table 3). The EC_{50} for LNCaP cells,

Scheme 1

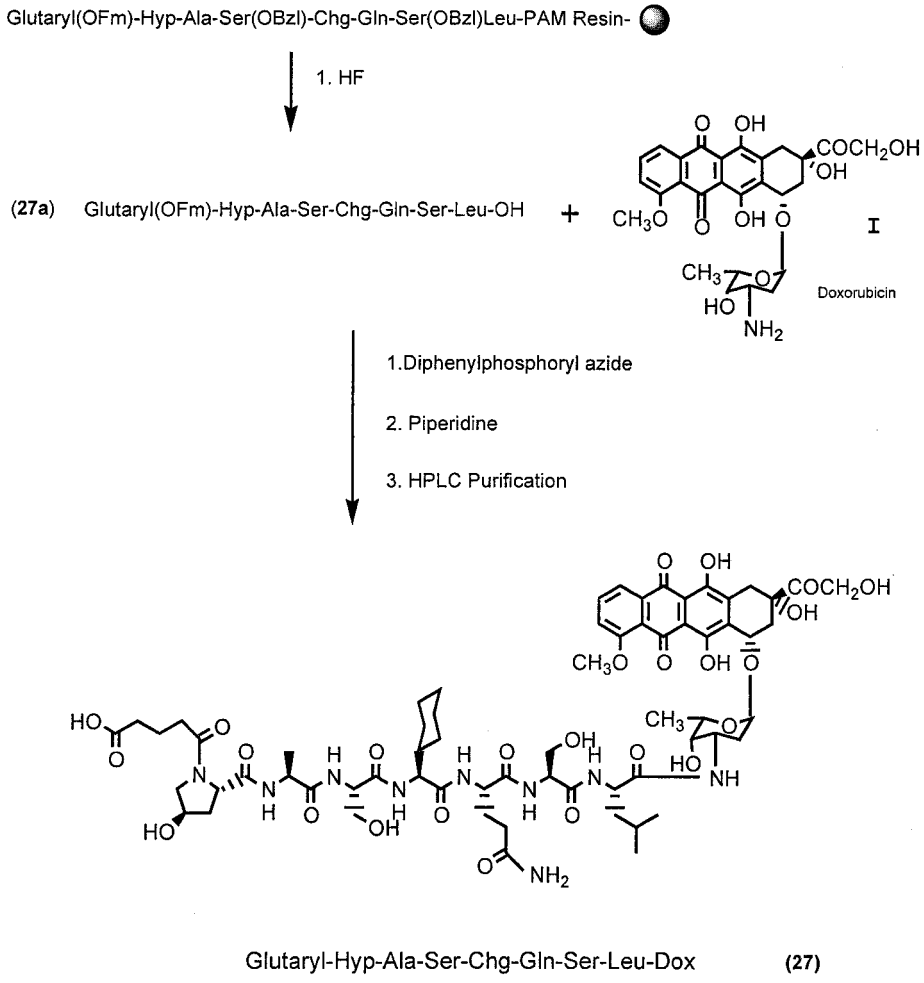

Table 3. Biological Data

		in vitro			in vivo			
	doxorubicin-conjugate structure	$T_{1/2}$[a] min	EC_{50} (μM) LNCaP[b]	EC_{50} (μM) DuPRO[c]	% PSA[d] reduction*	% tumor[d] wgt reduc.**	dose (μmol/kg)	MTD[f] (μmol/kg)
	doxorubicin (Dox)		0.7	1.8	16	6	2.8	2.8
12	H-Ser-Leu-Dox[g]							
13	H-Leu-Dox		6.7	14.0	10	(5)[e]	7.6	7.6
14	Ac-Ala-Ala-Ala-Dox		>100	>100	ND			
15	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ser-Ser-Leu-Dox	30	7.0	117	ND			
16	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ala-Ser-Leu-Dox	30	12.5	>100	ND			
17	Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ser-Leu-Dox	100	6.3	>100	87	81	36.6	>36.6
18	Ac-Lys-Tyr-Gln-Ser-Ser-Ser-Leu-Dox	60	6.9	>100	90	70	29.0	32.6
19	Ac-Orn-Tyr-Gln-Ser-Ser-Ser-Leu-Dox	120	7.0	>100	93	80	29.3	33.0
20	Ac-Lys-Ala-Ser-Tyr-Gln-Ser-Leu-Dox	30	4.8	100	ND			
21	Ac-Orn-Ala-Ser-Tyr-Gln-Ser-Leu-Dox	40	7.3	>100	70	57	14.8	29.7
22	Ac-Orn-Ala-Ser-Tyr-D-Gln-Ser-Leu-Dox	>1200	85	>100	22	26	18.9	>45.3
23	Ac-Lys-Ala-Ser-Chg-Gln-Ser-Leu-Dox	10	4.5	>100	95	73	22.4	29.9
24	Ac-Pro-Ala-Ser-Chg-Gln-Ser-Leu-Dox	45	7.4	>100	ND			
25	Ac-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	20	4.5	>100	96	81	22.7	22.7
26	Succinyl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	55	5.7	100	94	72	21.7	28.9
27	Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox	30	5.0	>100	95	87	21.5	28.6

[a] $T_{1/2}$ = time required for PSA to hydrolyze 50% of compound at a molar ratio of 1:100, respectively. [b] LNCaP: PSA secreting human prostate cancer cell line. [c] DuPRO: nonsecreting PSA human prostate cancer cell line. [d] % PSA and % tumor weight reduction relative to vehicle control following intraperitoneal injection (ip) of conjugate (dose) for 5 consecutive days to nude mice bearing LNCaP tumor xenografts. [e] 5% increase in tumor weight ($P = 0.925$). [f] MTD, defined as maximum nonlethal drug dose which could be administered (ip) to nontumor bearing nude mice for 5 consecutive days. [g] Compound was not tested due to its insolubility. *P (values): Dox (0.508); **13** (0.360); **17** (0.009); **18** (0.007); **19** (0.001); **21** (0.016); **22** (0.112); **23** (0.001); **25** (<0.001); **26** (<0.001); **27** (<0.001). **P (values): Dox (0.868); **13** (0.925); **17** (0.006); **18** (0.002); **19** (0.002); **21** (0.006); **22** (0.518); **23** (0.016); **25** (0.001); **26** (<0.001); **27** (<0.001).

which secrete PSA, was 7.0 μM while the EC_{50} for the non-PSA secreting DuPRO cell line was 117 μM. Leu-Dox shows comparable cell kill activity against LNCaP cells, EC_{50} = 6.7 μM, with little selectivity vs DuPRO cells, EC_{50} = 14.0 μM. Doxorubicin was also shown to be minimally selective against the two cell lines. These

results provide clear evidence that one can achieve selective targeting, in vitro, of PSA secreting human cancer cells through the use of a peptide-doxorubicin conjugate.

Our objective was to further show efficacy against PSA secreting tumors in vivo. Continued SAR studies focused on the development of peptide-Dox conjugates having a minimum molecular weight with the following properties: high selective potency against PSA secreting cells, a rapid specific rate of hydrolysis by PSA, resistance to nonspecific proteolysis, aqueous solubility, and efficacy against PSA-secreting tumors.

The SAR studies were continued with Ala substitution for Ser at P2′ to give **16**. This analogue, **16**, did not provide an improvement in the $T_{1/2}$ relative to **15** as did a comparable double Ala (P4, P2′) replacement in the peptide, **11** (Table 1). Truncation of a P′ Ser in **15** produced **17** with a 3-fold loss in the $T_{1/2}$; however, its selectivity profile against PSA secreting LNCaP cells remained. Continued deletion studies combined with point substitution provided two series of 7-mers having a favorable profile of in vitro cell kill and in vivo antitumor activity (Table 3). Coincident with the deletion studies, we introduced the unnatural amino acid cyclohexylglycine, Chg, as a substitution for Tyr at P2. This substitution was designed to stabilize the conjugates to undesired proteolytic hydrolysis by chymotrypsin-like proteases in plasma. Gratifyingly the conjugates with Chg as a substitution for Tyr maintained their selective PSA cleavage profile and in some cases had an improved $T_{1/2}$ as in the case of **23** vs **20** (Table 3).

The antitumor activity of these conjugates as well as Leu-Dox and doxorubicin was determined by assessing the ability of each compound to suppress the growth of human prostate cancer cell (LNCaP) xenografts in nude mice. Serum PSA levels and tumor weights were determined at the completion of the study. Table 3 reports the percent PSA reduction relative to vehicle control following conjugate treatment. Tumor weight loss correlates directly with the level of PSA reduction. Details describing the experimental biology were recently reported (also Table 3 footnotes).[7]

The first series of 7-mers as represented by **18** and **19** have an extended four amino acid P′ region. The second series of 7-mers, starting with **20**, have only two amino acids, Ser and Leu, in P′ linking doxorubicin. We believe that the shorter P′ may, in principle, offer a biological advantage in that minimal processing post PSA cleavage is necessary to liberate active cytotoxic Leu-Dox and doxorubicin from the intermediate H-Ser-Leu-Dox (**12**).

Both series of conjugates are potent inhibitors of tumor growth. However, we discovered that the extended P′ series (**18**, **19**) produced an unexpected and undesirable hypotensive effect which was due to histamine release following iv administration in dogs (data not shown). Interestingly neither doxorubicin nor the unconjugated peptide, Ac-Lys-Tyr-Gln-Ser-Ser-Ser-Leu-NH_2, displayed the histamine release associated with the conjugates (**18**, **19**). It has been reported that peptides with a hydrophobic functionality in combination with basic amino groups can sometimes cause histamine release from mast cells, and approaches to minimizing this effect have been described.[18,19] We were able to eliminate the histamine effect in the dipeptidyl P′ series by substituting the basic Lys or Orn at P5 with the secondary amino acid Pro (**24**) without compromising cytotoxic activity (Table 3). However, with the removal of the positive charge associated with Lys or Orn, the aqueous solubility of **24** was reduced to the degree that further development was difficult. While the introduction of the hydrophilic amino acid Hyp (**25**) provided a conjugate with improved solubility, it was not quite soluble enough for iv formulation. The introduction of a succinyl group at the N-terminus, in place of the acetyl group (affording **26**), was designed to improve solubility. While good aqueous solubility was achieved, it resulted also in an increase in the $T_{1/2}$ from 20 min (**25**) to 55 min (**26**). We found, however, that use of a glutaryl group afforded **27** which displayed both good solubility and a $T_{1/2}$ of 30 min.

The property of being a substrate for PSA is a requirement for antitumor activity of **27** if it functions by the proposed biological mechanism underlying this concept. An additional test of this hypothesis was to prepare a peptide-doxorubicin conjugate that was not a good substrate for PSA and to test that compound for antitumor activity against PSA-producing tumors in vivo. By incorporating the D-stereoisomer of Gln, the structurally related conjugate, Ac−Orn-Ala-Ser-Tyr-D-Gln-Ser-Leu-Dox (**22**), was specifically designed not to be a substrate for cleavage by PSA. In fact **22** failed to show any evidence of cleavage on exposure to PSA for up to 4 h (Table 3). Additionally, when tested in tumor xenograft studies, **22** was no more effective than doxorubicin against PSA secreting tumors.[7]

From these studies **27** emerged as the peptide-doxorubicin conjugate with the best profile of physical and biological properties. Compound **27** has a greater than 20-fold selectivity against human prostate PSA-secreting LNCaP cells relative to the non-PSA-secreting DuPRO cell line (Table 3). In nude mouse xenograft studies using LNCaP prostate cancer cells, **27** reduced PSA levels by 95% and tumor weight by 87% at a dose below its MTD (Table 3). Both doxorubicin and Leu-Dox (**13**) were ineffective in reducing circulating PSA and tumor burden at their maximum tolerated doses (Table 3).

Summary

The initial steps in designing the potentially useful doxorubicin prodrug, **27**, were 2-fold: first to identify a functionality of doxorubicin that when coupled to a peptide fragment would yield a biologically inactive (i.e., noncytotoxic) compound, and second to identify a peptide which, when coupled to doxorubicin, would be converted by PSA into the active drug, doxorubicin. The coupling of the tripeptide, $Ac(Ala)_3$-OH, with **I** to form **14** satisfied the first of the above criteria.

The identification of an appropriate substrate resulted from cleavage maps following PSA treatment of PSA's physiological substrate, semenogelin I. The most efficient cleavage site from recombinant semenogelin I occurs between Gln^{349} and Ser^{350}. Systematic modification, through the use of chemically synthesized 19 component peptide libraries and point substitution SAR studies of the amino acid residues flanking this cleavage

site, led to the design of short peptides which were efficiently hydrolyzed by PSA.

Doxorubicin-peptide conjugates were prepared based on, in part, structural data derived from the peptide optimization studies described above. Synthesis of the peptide-doxorubicin conjugates utilized a combination of solid-phase and solution chemistries.

The conjugates were optimized for maximal PSA $T_{1/2}$ cleavage rates, selectivity for PSA-secreting tumor cells, and efficacy in nude mouse xenograft studies using PSA-secreting human LNCaP prostate cancer cells. From these studies **27** was found to have the most favorable profile of physical and biological properties.

Compound **27** is effectively digested by the human PSA enzyme with a $T_{1/2}$ of 30 min while resisting digestion by human proteases in whole blood or plasma (data not shown). Compound **27** exhibits a >20-fold selectivity for killing PSA-producing prostate cancer cells versus non-PSA producing cancer cells in cell culture (Table 3). In nude mouse xenograft studies using human LNCaP prostate cancer cells that secrete PSA, **27** demonstrates dramatically improved antitumor activity compared to doxorubicin or Leu-Dox as measured by circulating PSA levels and tumor weights (Table 3).[7] Additionally, **22**, which is a peptide−doxorubicin conjugate that is not cleaved by PSA, was evaluated and found not to exhibit antitumor activity more effective than free doxorubicin against LNCaP tumors (Table 3).[7]

The PSA peptide−doxorubicin conjugate, **27**, is intended as an intravenous anticancer agent for the treatment of hormone refractory prostate cancer. Compound **27** is a prostate-targeted prodrug that is itself less toxic than the parent drug until activated by proteolytic cleavage to its active moieties, leucyl-doxorubicin (Leu-Dox) and doxorubicin (Dox). The intial step in this activation is preferentially carried out by the serine protease PSA which is expressed in prostate epithelial tissue but is not found at significant levels in other tissues. Additional data supporting tumor selective delivery of doxorubicin by the PSA targeted peptide conjugate, **27**, in a nude mouse xenograft model of human prostate cancer, was recently presented.[20] In theory, **27** should circulate freely in the body and be preferentially activated at sites of prostate cancer tissue, including metastatic foci, by PSA. Therefore, **27** should exhibit better antitumor activity against prostate cancers than free doxorubicin without enhanced toxicities.

Experimental Section

Abbreviations. Abbreviations for common amino acids are in accordance with recommendations of IUPAC. Other abbreviations: Boc, *tert*-butyloxycarbonyl; Bom, benzyloxymethyl; Br-Z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Chg, L-cyclohexylglycine; Cl-Z, 2-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DPPA, diphenylphosphoryl azide; Fmoc, fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazole *N*′,*N*′,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBT, *N*-hydroxybenzotriazole; Hyp, *trans*-4-hydroxy-L-proline; NMP, *N*-methylpyrrolidone; OcHex, *O*-cyclohexyl; OFm, fluorenylmethyl; Orn, L-ornithine; PAM, 4-(oxymethyl)-phenylacetamidomethyl; TEA, triethylamine; TFA, trifluoroacetic acid; Tos, tosyl.

Preparation of Semenogelin I and the Determination of PSA Cleavage Sites. Semenogelin I cDNA was cloned from total mRNA that had been isolated from human prostate tissue (CLONTECH, Palo Alto, CA) using deoxyoligonucleotide primers whose sequence was taken from the published sequence of human semenogelin I. The semenogelin I cDNA was tagged with a tubulin epitope (glu-glu-phe) at the 3′ end and placed in the bacterial expression vector pGEX 2T. Semenogelin I protein production was induced with the addition of IPTG. Semenogelin I was purified to greater than 95% homogeneity using a glutathione affinity column. The eluate was digested with thrombin to remove the glutathione transferase leader and the digest purified using an immunoaffinity column containing the anti-tubulin antibody YL1/2. The recombinant semenogelin I protein was used as a substrate for PSA that was purified from human seminal fluid (York Biologicals Inc., StonyBrook, NY). The proteolysis reaction contained recombinant semenogelin I and PSA in a molar ratio of 100 to 1, respectively, in 20 mM TRIS·HCl, 25 mM NaCl, 0.5 mM $CaCl_2$, at pH 7.2. The proteolysis reaction was incubated for 5 min and terminated by the addition of gel sample buffer and heating to 100 °C for 5 min. The proteolytic digest was resolved by SDS polyacrylamide gel electrophoresis and transferred via electroblotting to a PVDF membrane. Regions of the PVDF membrane containing protein were cut out and submitted for amino acid sequence analysis (W. M. Keck Foundation, Biotechnology Resource Laboratory, Yale University, New Haven, CT). Five unique amino temini were identified and are indicated as CS-1 (cleavage site number 1) through CS-5 in the Supporting Information figure. The cleavage sites in the Supporting Information figure are numerically ranked according to the number of nanomoles of amino acid residues generated during the 5 min cleavage reaction, with CS-1 representing the most common species found.

***$T_{1/2}$* Determination: Time to 50% Cleavage of Peptides and Peptide-Dox Conjugates by Purified PSA.** Oligopeptides were mixed with PSA at a molar ratio of 100 to 1, respectively, in 50 mM TRIS·HCl and 140 mM NaCl at pH 7.4. The reaction was incubated for 0, 30, 60, 120, 180, or 240 min at 37 °C. The reactions were quenched with $ZnCl_2$ (10 mM final concentration), and the products were analyzed by HPLC on a Vydac C_{18} column using a 5% to 50% gradient of 0.1% aqueous TFA−acetonitrile. The chromatograms were monitored at 210 nM and 479 nM in order to follow the peptide and doxorubicin components, respectively, of the peptide-doxorubicin conjugates. The differential area of the substrate peak versus the area of the untreated prodrug peak was used as a measurement of peptide hydrolysis. Results are reported as the time required for PSA to hydrolyze 50% of the initial substrate.

General Methods for Synthesizing Peptides and Protected Peptide Intermediates. All of the peptides were synthesized by Merrifield solid-phase peptide synthesis using a double-coupling protocol on the Model 430A Applied Biosystems peptide synthesizer (Foster City, CA). The resin for the synthesis of carboxy terminal peptide amides was *p*-methylbenzhydrylamine (MBHA) and 4-(oxymethyl)-phenylacetamidomethyl (PAM) for carboxy terminal peptides (purchased from ABI). N^{α}-Boc-protected amino acids of the L-configuration and reagents were supplied by the manufacturer. Protected N^{α}-Boc amino acids of the D-configuration as well as N^{α}-Fmoc amino acids were purchased from Bachem or prepared by standard methods. Side-chain protection was Arg (Tos), Asp (OcHex), Glu(OcHex), His(Bom), Lys(Cl-Z), Orn(Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(Br-Z). Amino acids were coupled in NMP as a solvent using dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBT). ABI software version 1.40 for HOBT/NMP cycles was used starting with 0.5 mmol of resin and a 4-fold excess of activated protected amino acid. Deprotection and removal of the peptides from the resin support were effected using anhydrous HF with 10% anisole at 0° for 1 h.[21] The Fmoc and fluorenyl methyl ester (OFm) protecting groups were not removed by HF. Peptides were purified by preparative high-pressure liquid chromatography (HPLC) (Separations Technology) on reverse-phase radial compression C_{18} columns (Waters, Delta-Pak, 15 μm, 100 Å, 5 × 30 cm). A step gradient (5%A to 50%A) (100 mL increments) was generated from 1 L each of successively increasing concentrations of mobile phase

(solvent A, 0.1% trifluoroacetic acid/H_2O; solvent B, acetonitrile/0.1% trifluoroacetic acid). A flow rate of 80 mL/min was used to elute the peptides. Detection was performed by monitoring the UV absorbance at 214 nm. Homogeneous product fractions (>98% pure) were pooled and freeze-dried. The homogeneity of the peptides was demonstrated by analytical HPLC using a Vydac 15 cm, 218TP5415, C_{18} column. Identity was confirmed by amino acid compositional analysis following HCl hydrolysis (results not shown) and mass spectral analysis.

Mass Spectral Analysis. Mass measurements of the compounds were conducted on one of the following three instruments: VG Autospec -Q by use of electrospray (ES) or fast atom bombardment (FAB) ionization; VG ZAB-HF by use of FAB; or the Bruker BioApex 7T FT/ICR/MS by use of ES. Unless otherwise noted, the *m/z* values are reported as $[M+H]^+$ (Supporting Information table).

Synthesis of Peptides 1, 2, 3, 4. The title compounds were individually prepared by solid-phase methods as outlined above, starting with 0.5 mmol of C-terminal N-Boc-protected amino acid PAM resin. The subsequent protected amino acids were coupled using standard ABI protocols as described above. Following completion of the assembly of the resin bound peptides, the N-terminal Boc-protecting groups were removed using the TFA deblocking cycle. Cleavage and deprotection of the peptides were effected using the following standard HF procedure. To the dried resin was added anisole (1 mL/g resin) as a scavenger. The vessel was cooled with liquid nitrogen and evacuated. Anhydrous liquid HF was distilled into the reaction (9 mL/g of resin) following which the mixture was stirred for 1 h at 0 °C. The HF was removed by vacuum distillation. The residue was triturated with ethyl ether, filtered, and washed 3× with additional ether. The peptide was extracted with 10% acetic acid 3× (15 mL/g of resin) and the filtrate lyophilized to give the desired crude product. Purification of the peptides was achieved by preparative RP-HPLC as described above. A sample of crude peptide (400−500 mg) was dissolved in 50 mL of 5% acetic acid and subsequently pumped onto a preparative HPLC column which had been equilibrated in 5% starting buffer (additional details in general methods above). Homogeneity of the peptides was demonstrated by analytical RP-HPLC. See Supporting Information table for analytical data for the purified peptides.

Synthesis of Peptides 5, 6, 7, 8, 9, 10, 11. Starting with 0.5 mmol of MBHA resin to provide the final product in the C-terminal amide form, the title compounds were prepared as reported above. The N-terminal acetyl group was introduced by coupling acetic acid, 2 mmol (4 equiv). Cleavage and deprotection of the resin bound peptides were effected using HF as described above. Lyophilized crude peptides were purified by preparative HPLC as described above.

Synthesis of Peptide Libraries 1L, 2L, 3L, 4L. The mixed peptide libraries were prepared and characterized as described in a previous report.[15] In brief, the primary protected dodecapeptide sequences were assembled on 0.5 mmol MBHA resin using the ABI synthesizer. For the mixed position, Xaa, a manual coupling was carried out with one equivalent of an equimolar mixture of 19 Boc-protected amino acids (Table 2) which was dissolved in NMP and coupled using 1.05 equiv (0.525 mmol) of HBTU and HOBT and 2.1 equiv of DIEA. The reactions were allowed to proceed to completion (24 h) as monitored by ninhydrin. Deprotection and cleavage were performed with anhydrous HF as described above using 10% *p*-cresol/*p*-thiocresol, 1:1 (v/v), as scavengers. After removal of the HF and scavengers in vacuo, the free peptides were precipitated with diethyl ether, filtered, and extracted with 50% aqueous acetic acid, diluted with H_2O, and lyophilized providing the 19-component peptide libraries.

H-Ser-Leu-Dox, 12. Synthesis of the titled compound was from the dipeptide intermediate, Fmoc-Ser-Leu-OH (**12a**), which was prepared by solid-phase chemistry as described in example **15**. Fmoc-Ser(tBu) was coupled to H-Leu-PAM resin followed by HF cleavage to provide, after workup, **12a**. Reaction conditions for the coupling of **12a** with doxorubicin were as described in **19**. Purification conditions were as described for example **18**.

Leu-Dox, 13. Fmoc-Leu (182 mg, 0.515 mmol) was dissolved in DMSO (6 mL) to which was added HOBT (1.70 mL of a 1 M solution in NMP) followed by DCC (1.55 mL of a 1 M solution in NMP). The solution was stirred at room termperature for 30 min followed by the addition of 200 mg of doxorubicin hydrochloride (0.35 mmol) dissolved in DMSO (3 mL). The solution was cooled to 5 °C and the pH adjusted with DIEA (2M in NMP) to ∼7 using indicator strips (pH 0−14, EM Science). After being stirred for 18 h at 5−10 °C the reaction was transferred to a 200 mL round-bottomed flask, cooled to 0 °C, and to it added 4 mL of piperidine for the deblocking of the Fmoc protecting group. The reaction was evaporated in vacuo and the oil dissolved in 200 mL of a buffer consisting of 80% ammonium acetate (0.1% in H_2O), (A) and 20% acetonitrile (B). The solution containing the product was purified by RP-HPLC on a C_{18} radial compression column (Waters, Delta Pak, 300 Å, 15 μm, 5 × 30 cm) which had been equilibrated in 80:20 (A:B). A step gradient was generated (100 mL increments) from 1 L each of successively increasing (5%) concentrations of mobile phase (20% to 40%). A flow rate of 80 mL/min was used to elute the product. The elution was monitored at 260 nm. The pure fractions were pooled and freeze-dried. The dried product was dissolved in 200 mL of 1% acetic acid and freeze-dried again to remove possible traces of ammonium acetate. Purity of the product was checked by analytical RP-HPLC and determined to be >98%.

Ac-Ala-Ala-Ala-Dox, 14. Ac-Ala-Ala-Ala-OH (Sigma) (4.6 mg, 12.3 μmol) and doxorubicin hydrochloride (9.7 mg, 16.8 μmol) were dissolved in DMSO (1 mL). To the stirred solution was added 11.4 mg HOBT (84 μmol) and 31.9 mg (84 μmol) HBTU in DMSO (1 mL) followed by 8.6 μL (89 μmol) of DIEA. The solution was stirred at ambient temperature overnight. The reaction was found to have gone to completion as determined by analytical RP-HPLC (conditions found in footnote c, Supporting Information table). The reaction was diluted with a 1% acetic acid aqueous solution (8 mL) and purified by semipreparative RP-HPLC (C_{18} Vydac, 218TP 1022), elution at 8 mL/min, with a linear gradient of 0.1% aqueous TFA−acetonitrile from 90:10 to 10:90 over 60 min with detection at 214 nm. Fractions with the desired purity (>98%) were pooled and lyophilized.

Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ser-Ser-Leu-Dox, 15. The primary peptide sequence was prepared on a 0.5 mmol scale as described above. Lysine was introduced as Boc-Lys (Fmoc)-OH. Following HF cleavage and ether precipitation, the protected peptide intermediate was extracted with DMSO (3 × 50 mL) and the DMSO removed in vacuo. The residue was triturated with H_2O, filtered and dried in vacuo to provide Ac-Ala-Asn-Lys (Fmoc)-Ala-Ser-Tyr-Gln-Ser-Ser-Ser-Leu-OH (**15a**). Compound **15a** was used without purification by dissolving 113 mg (0.080 mmol) in DMSO (2 mL), and to the solution was added HOBT (3.3 equiv, 0.264 mL of a 1 M solution in NMP) followed by DCC (3.3 equiv, 0.264 mL of a 1 M solution in NMP). After the mixture stirred for 30 min, doxorubicin hydrochloride (46.4 mg, 0.08 mmol) in DMSO (0.8 mL) was added followed by the addition of DIEA to a pH of ∼7 (pH indicator paper). After the reaction mixture was stirred for 18 h at 5−10 °C, the Fmoc protecting group was removed at 0 °C by the addition of 3 mL of piperidine. Following 1 min at 0 °C the reaction was evaporated in vacuo, the resulting residue was suspended in 400 mL of aqueous NH_4HCO_3 (0.1%) and filtered, and the filtrate was purified as described above by preparative HPLC on a Delta Pak C_{18} column which had been equilibrated with 100% A buffer (0.1% NH_4HCO_3). A step gradient was generated (100 mL increments) from 1 L each of successively increasing (5%) concentrations of mobile phase (B = acetonitrile). Pure fractions (>98%) were pooled and lyophilized. The dried product was dissolved in 1% acetic acid and relyophilized.

Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ala-Ser-Leu-Dox, 16. Synthesis of the intermediate, Ac-Ala-Asn-Lys(Fmoc)-

Ala-Ser-Tyr-Gln-Ser-Ala-Ser-Leu-OH (**16a**), and the titled compound was as described for **15**.

Ac-Ala-Asn-Lys-Ala-Ser-Tyr-Gln-Ser-Ser-Leu-Dox, 17. Synthesis of the intermediate, Ac-Ala-Asn-Lys(Fmoc)-Ala-Ser-Tyr-Gln-Ser-Ser-Leu-OH (**17a**), and the titled compound was as described for **15**.

Ac-Lys-Tyr-Gln-Ser-Ser-Ser-Leu-Dox, 18. The intermediate for the titled compound, Ac-Lys(Fmoc)-Tyr-Gln-Ser-Ser-Ser-Leu-OH (**18a**), was prepared by automated solid-phase synthesis as described in example **15**. Compound **18a** (0.214 mg, 0.2 mmol) was dissolved in 4 mL of DMSO. To the stirred solution was added 2.64 equiv of HOBT (0.53 mL of a 1 M solution in NMP) and 2.40 equiv of DCC (0.48 mL of a 1 M solution in NMP). After 30 min doxorubicin hydrochloride was added (100 mg, 0.172 mmol) in 2 mL of DMSO followed by the dropwise addition of DIEA to adjust the pH ∼ 7 (pH indicator paper). After the mixture was stirred at room temperature overnight, 1 mL of DMSO was added and the reaction cooled to 0 °C followed by the addition of piperidine (3 mL) to remove the Fmoc protecting group. After 1 min the reaction was evaporated in vacuo. The crude solid was suspended in 150 mL of a 90:10 (A:B) solution where buffers A = 15% acetic acid in H_2O and B = 15% acetic acid in methanol. Following filtration, the filtrate which contained the product was applied to a Delta Pak C_{18} preparative HPLC column and the product eluted as described earlier using the above sample dissolution buffers. The column was equilibrated with 90:10 (A:B) and the product eluted using a step gradient (100 mL increments) from 1 L of the following successively increasing concentrations of A:B: 80:20, 70:30, 65:35, 60:40, 55:45, 50:50. Pure (>98%) product fractions were pooled, concentrated in vacuo, and lyophilized from water.

Ac-Orn-Tyr-Gln-Ser-Ser-Ser-Leu-Dox, 19. Synthesis of the intermediate, Ac-Orn (Fmoc)-Tyr-Gln-Ser-Ser-Ser-Leu-OH (**19a**) was as described for **15a** in example **15**. To a stirred solution of **19a** (212 mg, 0.2 mmol) in 8 mL of DMF was added doxorubicin hydrochloride (100 mg, 0.172 mmol) followed by the addition of 20 μL of TEA. The solution was cooled to 0 °C and to it added 24 μL of DPPA (0.55 equiv, 0.11 mmol) followed by a second addition of DPPA (24 μL) after 5 min. The pH of the reaction was adjusted to ∼7 (pH indicator paper) with TEA. After being stirred at 0 °C for 90 min, additional DPPA (24 μL) was added and the pH readjusted to ∼7 with TEA. Following stirring at 0 °C overnight, DMF (1 mL) was added followed by 3 mL of piperidine. The removal of the Fmoc protecting group was allowed to proceed at 0 °C for 1 min and the reaction concentrated to an oil in vacuo. Purification of the crude to provide pure (>98%) product, **19**, was as described for **18**.

Ac-Lys-Ala-Ser-Tyr-Gln-Ser-Leu-Dox, 20. Synthesis of the titled compound was as described for **19** from the intermediate Ac-Lys(Fmoc)-Ala-Ser-Tyr-Gln-Ser-Leu-OH (**20a**).

Ac-Orn-Ala-Ser-Tyr-Gln-Ser-Leu-Dox, 21. Synthesis of the titled compound was as described for **19** from the intermediate Ac-Orn(Fmoc)-Ala-Ser-Tyr-Gln-Ser-Leu-OH (**21a**).

Ac-Orn-Ala-Ser-Tyr-D-Gln-Ser-Leu-Dox, 22. Synthesis of the titled compound was as described for **19** from the intermediate Ac-Orn(Fmoc)-Ala-Ser-Tyr-D-Gln-Ser-Leu-OH (**22a**).

Ac-Lys-Ala-Ser-Chg-Gln-Ser-Leu-Dox, 23. Synthesis of the titled compound was as described for **19** from the intermediate Ac-Lys(Fmoc)-Ala-Ser-Chg-Gln-Ser-Leu-OH (**23a**).

Ac-Pro-Ala-Ser-Chg-Gln-Ser-Leu-Dox, 24. Synthesis of the titled compound was as described for **19** from the intermediate Ac-Pro-Ala-Ser-Chg-Gln-Ser-Leu-OH (**24a**).

Ac-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox, 25. The intermediate for the titled compound, Ac-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH (**25a**), was prepared by solid-phase synthesis as described above. Boc-Hyp was introduced without protection of the hydroxyl side chain. Reaction conditions for the coupling with doxorubicin were as described in example **19**. Purification conditions were as described for example **18**.

Succinyl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox, 26. The intermediate for the titled compound, succinyl(OFm)-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH (**26a**), was prepared by solid-phase synthesis as described above. The introduction of the succinyl group was via coupling of the monofluorenylmethylester of succinic acid. Introduction of doxorubicin was as described for example **19**. Removal of the fluorenylmethyl protecting group was achieved by treatment with piperidine as described in example **15**. Purification by preparative HPLC was as described for example **18**.

Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox, 27. The intermediate for the titled compound, Glutaryl(OFm)-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH (**27a**), was prepared by automated solid-phase synthesis as described in Example **15**. The glutaryl functionality was introduced as the monofluorenylmethylester of glutaric acid using the usual coupling protocol (general methods section). HF cleavage and workup was as described for **15**. Compound **27a** was used without purification by dissolving 170 mg (0.16 mmol) in DMF:DMSO, 1:1 (20 mL). To the stirred solution was added 80 mg of doxorubicin hydrochloride (0.14 mmol) followed by 50 μL of DIEA (0.28 mmol). The solution was cooled (0 °C) and to it added DPPA, 43 μL (0.20 mmol). After 30 min an additional 43 μL of DPPA was added and the pH adjusted to ∼7 (pH indicator paper). After 4 h the reaction was shown to be complete by analytical HPLC. DMF (10 mL) was added followed by piperidine (3.5 mL). After 1 min at 0 °C the solution was concentrated in vacuo to an oil. The crude product was triturated with diethyl ether and filtered. The dried crude was dissolved in 250 mL of a buffer consisting of 80% ammonium acetate (0.1% in H_2O) (A) and 20% acetonitrile (B). The product was purified by RP-HPLC on a C_{18} radial compression column which had been equilibrated in 80:20 (A:B). A step gradient was generated (100 mL increments) from 1 L each of successively increasing (5%) concentrations of mobile phase (20% to 40%). A flow rate of 80 mL/min was used to elute the product. Pure fractions were pooled and lyophilized. The dried product was dissolved in 1% acetic acid and lyophilized again to remove possible traces of ammonium acetate. Purity of the product (90 mg, 40% yield from **27a**) was checked by analytical HPLC and determined to be >98%.

Supporting Information Available: Figure containing amino acid sequence of human semenogelin I and table containing synthetic peptides and doxorubicin conjugates analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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