

Enzymatic Activation of a Doxorubicin-Peptide Prodrug by Prostate-Specific Antigen¹

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Abstract

New approaches to target cytotoxic therapy specifically to metastatic prostate cancer sites are urgently needed. As such an approach, an inactive prodrug was synthesized by coupling the primary amine of doxorubicin to the COOH-terminal carboxyl of a seven-amino acid peptide carrier (*i.e.*, Mu-His-Ser-Ser-Lys-Leu-Gln-Leu). The seven-amino acid peptide was documented to be hydrolyzable specifically by the serine protease prostate-specific antigen (PSA) to liberate the active cytotoxin L-leucyl-doxorubicin. Primary cultures of PC-82 human prostate cancer cells secreted high levels of enzymatically active PSA (*i.e.*, 70 ± 5 ng of enzymatically active PSA/ 10^6 cells/24 h), whereas LNCaP human prostate cancer cells produced lower levels of enzymatically active PSA (*i.e.*, 2.3 ± 1 ng/ 10^6 cells/24 h). LNCaP cells, however, secreted sufficient amounts of enzymatically active PSA to activate the doxorubicin prodrug to a cytotoxic form *in vitro*. The specificity of the cytotoxic response to the prodrug was demonstrated by the fact that 70 nM of the prodrug killed 50% of the PSA-producing LNCaP cells, whereas doses as high as 1 μ M had no cytotoxic effect on PSA-nonproducing TSU human prostate cancer cells *in vitro*.

Introduction

There is currently no effective therapy for men with metastatic prostate cancer with relapse after androgen ablation, even though numerous agents have been tested over the past 30 years (1). Prolonged administration of effective concentrations of standard chemotherapeutic agents is usually not possible because of dose-limiting systemic toxicities. Therefore, new strategies are needed to target cytotoxic agents specifically to sites of metastatic prostate cancer while avoiding systemic toxicity. One such approach would be to develop prodrugs that are inactive when given systemically but become activated when processed proteolytically within prostate cancer metastases. To accomplish this, primary amine-containing agents can be coupled through the amino group to form a peptide bond to a peptide carrier (2).

One such primary amine-containing agent that can be readily modified to a prodrug form is the anthracycline doxorubicin (3). As a single agent, doxorubicin appears to have one of the best response rates among the large number of agents tested in the treatment of prostate cancer (4, 5), but cardiotoxicity and myelotoxicity have limited its use, especially in elderly men. Modifying doxorubicin to an inactive prodrug form may allow prolonged administration with substantially reduced toxicity. To design this prodrug, doxorubicin was linked through its primary amine to a peptide carrier so that the peptide linkage between the cytotoxic agent and the peptide could be

specifically cleavable only by the proteolytic activity of PSA.³ PSA is a M_r 33,000 single-chain glycoprotein that, in men, is synthesized and secreted in large quantities by normal and malignant prostatic epithelial cells (5-7).

PSA is a serine protease with chymotrypsin-like substrate specificity (8-10) that is found in high concentrations (*i.e.*, mg/ml) in the seminal plasma, where the major proteolytic substrates for PSA are the gel-forming proteins in freshly ejaculated semen, Sg I and Sg II (9). On the basis of the PSA cleavage map for Sg I and II, a peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was identified that had a high degree of specificity for PSA (11). This substrate was used to demonstrate that prostate cancer cells secrete enzymatically active PSA into the extracellular fluid and that PSA becomes inactivated by serum protease inhibitors on entering the blood (11). To study the proteolytic processing of the doxorubicin prodrug by PSA, an *in vitro* assay was developed using the androgen-sensitive human prostate cancer cell line LNCaP, which is the only prostate cancer cell line available that continues to produce PSA on serial passaging (12). In the present study, the enzymatic activity of PSA secreted by LNCaP cells *in vitro* was directly measured using the HSSKLQ-AMC substrate. After characterizing the enzymatic activity of PSA produced by LNCaP cells *in vitro*, doxorubicin prodrugs were synthesized and tested for *in vitro* activity against the LNCaP cell line.

Materials and Methods

Cell Lines. The androgen-responsive LNCaP and androgen-independent TSU-Pr1 human prostate cancer lines were obtained from American Type Culture Collection (Manassas, VA) and maintained by serial passage in RPMI 1640 containing 10% FCS with 100 units/ml penicillin G and 100 units/ml streptomycin sulfate (antibiotics from M. A. Bioproducts, Walkersville, MD) as standard medium in 5% CO₂/95% air at 37°C. The origins and characteristics of the LNCaP and TSU-Pr1 cell lines have been described previously (12, 13). To collect PSA to assay enzymatic activity, LNCaP cells were grown in standard medium to 70-80% confluence. The serum-containing medium was then removed, cells were washed twice with HBSS, and then new serum-free medium was added that consisted of RPMI 1640 containing antibiotics and 100 nM dihydrotestosterone. PC-82 androgen-dependent human prostate cancer xenografts were maintained by serial passage in athymic nude mice (Charles River). The origins and characteristics of this xenograft have been described (14). Primary cultures of PC-82 were established as described (15).

Kinetic Analysis of Substrate Hydrolysis by PSA. Substrate hydrolysis was studied by measuring fluorescence change secondary to AMC release, as described previously (11).

PSA Immunoprecipitation. PSA concentration in conditioned tissue culture medium was determined using the Tandem-R PSA assay (Hybritech, San Diego, CA) according to the manufacturer's instructions. Samples were then diluted to equivalent PSA concentrations in PSA assay buffer. PSA immunoprecipitation was then performed using the mouse monoclonal anti-PSA anti-

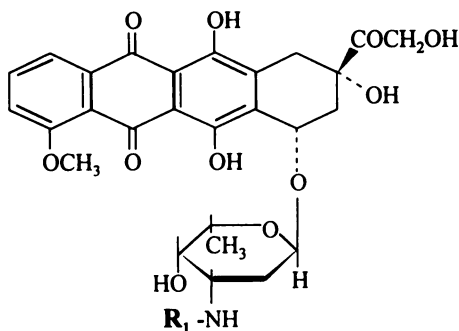
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³ The abbreviations used are: PSA, prostate-specific antigen; Sg, semenogelin; AMC, 7-amino-4-methyl-coumarin; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; ACT, α_1 -antichymotrypsin; A2M, α_2 -macroglobulin.



- When $R_1 = H$ = Doxorubicin (Dox)
 When $R_1 = \text{Ac-His-Ser-Ser-Lys-Leu-Gln}$ = Ac-HSSKLQ-Dox
 When $R_1 = \text{Mu-His-Ser-Ser-Lys-Leu-Gln-Leu}$ = Mu-HSSKLQ-Leu-Dox

Fig. 1. Doxorubicin-peptide prodrug structures.

body H117 that recognizes both free PSA and PSA complexed to ACT (16), as described previously (11).

Western Blotting. Samples containing 50 ng of PSA were separated by denaturing SDS-PAGE using 4–20% gradient gels (Bio-Rad) followed by transfer to Hybond-ECL nitrocellulose membranes (Amersham Corp.). Otherwise, the procedure was performed as described previously (11).

Sample Preparation. Conditioned medium from LNCaP cells containing the doxorubicin prodrug were applied to a C_{18} reversed-phase Bond-Elut column (Varian, Carpinteria, CA) and washed with 3 ml of buffer (*i.e.*, six column volumes) consisting of 0.1 M phosphoric acid and 5% acetonitrile in PBS as described (17). Samples were eluted from the column using 2 ml of a solution of 70% acetonitrile/0.1% TFA (*v/v*). The solvents were then evaporated to dryness, and the samples were redissolved in 0.1% TFA (*v/v*) and applied to the HPLC column.

Chromatography. The HPLC system consisted of a dual-pump (model 126; Beckman Instruments, Columbia, MD) with a manual injection valve (Rheodyne, Cotati, CA) fitted with a 1-ml injection loop. A reversed-phase C_{18} Ultrasphere analytical column (Beckman) 15 cm \times 4.6 mm (inner diameter) was used together with a 4.5 cm \times 4.6 mm (inner diameter). Ultrasphere reversed-phase guard column (Beckman). A gradient elution was performed consisting of eluent A [0.1% TFA (*v/v*)] and eluent B [70% acetonitrile/0.1% TFA (*v/v*)], with a gradient of 0–30% B over 5 min and then 30–60% B over 25 min, with a flow rate of 1 ml/min. A diode array detector (model 168; Beckman) was used to monitor the effluent at 480 nm. All analyses were conducted at ambient temperature. Data processing was performed using the Gold Chromatography Data System, version 1.0 (Beckman).

Cytotoxicity Assays. Percentage clonogenic survival of TSU-Pr1 (2×10^5 cells) following 48-h exposure to varying concentrations of doxorubicin prodrugs with or without exogenously added PSA was determined as described (18). To analyze the cytotoxicity of the doxorubicin prodrugs against LNCaP cells, these cells were exposed to varying concentrations of prodrugs for 72 h. Cells were then counted, and the percentage viable cells was determined by trypan blue exclusion using a hemocytometer. The dose that produced 50% cytotoxicity as compared to controls was then determined (*i.e.*, the LD_{50}) for both LNCaP and TSU cells.

Materials. The MU-HSSKLQ-AMC substrate was custom synthesized by Enzyme Systems Products (Dublin, CA) and characterized as described (11).

Doxorubicin (Dox) prodrugs [N-Ac-His-Ser-Ser-Lys-Leu-Gln-Dox (Ac-HSSKLQ-Dox), where Ac is acetyl, and His-Ser-Ser-Lys-Leu-Gln-Leu-Dox (Mu-HSSKLQ-Leu-Dox), where Mu is morpholinocarbonyl] were synthesized by coupling the primary amine of doxorubicin to the carboxyl group of the COOH-terminal amino acid [by one of us (A. N.) and California Peptide Research, Inc., Napa, CA; Fig. 1]. Purification of both compounds by HPLC yielded the trifluoroacetate salt (>98% purity). The peptide sequence was confirmed by amino acid analysis, and molecular weights were confirmed by mass spectroscopy.

Doxorubicin was from Pharmacia (Kalamazoo, MI). L-Leucyldoxorubicin was synthesized by A. N. PSA was purified from human seminal plasma as described (19), ACT, A2M, and other reagents were obtained from Sigma

Chemical Co. (St. Louis, MO). Mouse monoclonal IgG anti-PSA antibodies H117 and 5A10 were described previously (16).

Results and Discussion

Enzymatic Activity of PSA Produced by PC-82 Cells *In Vitro*.

The PC-82 human prostate cancer xenograft model continues to secrete large quantities of PSA on serial passage in nude mice. PC-82 tumors can be removed and grown as primary *in vitro* cultures in defined serum-free medium containing 10^{-10} M dihydrotestosterone. Medium from these PC-82 primary cultures was collected after 4 days of conditioning, and after concentrating the medium, the PSA concentration was determined using the Tandem R assay. These results demonstrated that PC-82 cells secrete 70 ± 5 ng of PSA/ 10^6 cells/24 h. This secreted PSA was then assayed for enzymatic activity using the HSSKLQ-AMC substrate and was found to have $99.8 \pm 6\%$ of the activity of similar concentrations of enzymatically active PSA purified from human seminal plasma. The enzymatic activity was nearly completely (>98%) inhibited by the PSA-specific mAb 5A10. These results indicate that PC-82 primary cultures secrete PSA in an enzymatically active form and that hydrolysis of the HSSKLQ-AMC was solely due to PSA activity.

Enzymatic Activity of PSA Secreted by LNCaP Cells *In Vitro*.

The LNCaP human prostate cancer cell line is androgen sensitive and is the only serially passageable cell line that continues to secrete PSA *in vitro*. Serum-free media were used to eliminate the excess protease inhibitors commonly found in serum that could inactivate any enzymatically active PSA produced by these cells and 100 nM DHT was added to achieve the highest possible yield of PSA. After 4 days of conditioning by the LNCaP cells, the medium was removed and concentrated 20-fold, and the PSA concentration determined using the Tandem R assay. These results demonstrated that under these conditions, LNCaP cells secrete 14 ± 1 ng of PSA/ 10^6 cells/24 h (*i.e.*, 5-fold less than secreted by PC-82 cells). Initially, these medium concentrates were directly assayed for enzymatic activity using the HSSKLQ-AMC substrate, and no detectable activity was seen. To increase the total amount of PSA in each assay, an additional immunoprecipitation step using the PSA-specific mAb H117 was performed. The specificity and efficiency of this immunoprecipitation has been previously validated using enzymatically active PSA purified from seminal plasma (11). After immunoprecipitation of PSA

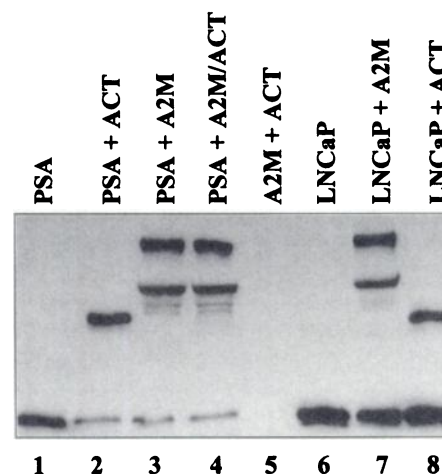


Fig. 2. Western blot analysis of PSA from LNCaP cells with protease inhibitors. Lanes 1–4, enzymatically active PSA purified from human seminal plasma incubated with 10-fold molar excess of protease inhibitors ACT and A2M. Lane 5, A2M and ACT controls (no PSA). Lanes 6–8, LNCaP concentrated PSA-containing medium incubated with indicated protease inhibitors. PSA samples (50 ng of total PSA/lane as determined by Tandem R assay) were incubated for 24 h at 37°C prior to analysis. The molecular weight of each band was determined by use of molecular weight marker standards.

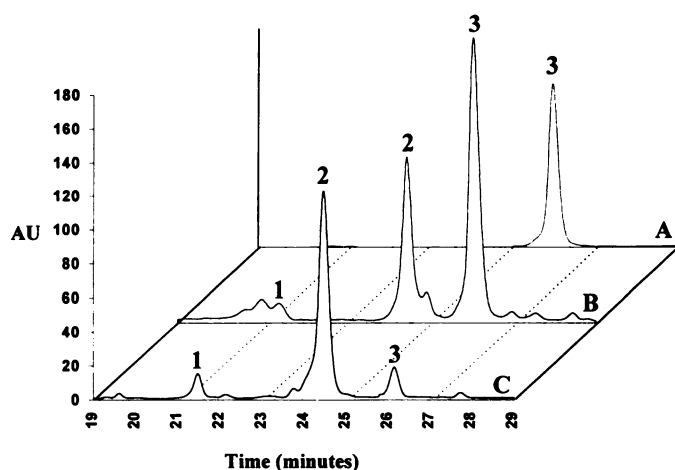


Fig. 3. HPLC analysis of proteolysis of doxorubicin prodrugs and by PSA. A, HSSKLQ-Dox, 0.4 mM incubated with 30 μ g/ml enzymatically active PSA for 24 h, and then 20 nmol of total doxorubicin were applied to the column. B, Mu-HSSKLQ-Leu-Dox, 0.4 mM incubated with 30 μ g/ml enzymatically active PSA for 24 h and then 40 nmol of total doxorubicin were applied to the column. C, spent medium from LNCaP cells treated with 10 μ M Mu-HSSKLQ-Leu-Dox for 72 h, followed by medium extraction as described, and then 20 nmol of total doxorubicin were applied to the column. Peak 1, doxorubicin; peak 2, Leu-Dox; peak 3, doxorubicin-peptide prodrugs. In each case, peaks were verified by use of purified standards.

from PC-82 primary cultures, 95 \pm 5% of the immunoprecipitated PSA from these samples retained enzymatic activity.

Following the immunoprecipitation step, the enzymatic activity from a total 1 μ g of PSA could be evaluated per assay, and therefore enzymatic activity can be detected, even if only 5% of the total PSA/assay is enzymatically active. When 1 μ g of total PSA was immunoprecipitated from the LNCaP-conditioned medium, it possessed only 16 \pm 1.5% of the enzymatic activity of similar concentrations of immunoprecipitated PSA purified from seminal plasma (*i.e.*, the LNCaP secreted 2.3 \pm 1 ng of enzymatically active PSA/10⁶ cells/24 h). Similar to the enzymatic activity of the purified seminal plasma PSA immunoprecipitate, the enzymatic activity of the PSA immunoprecipitated from the LNCaP medium was inhibited by >98% by the neutralizing antibody mAb 5 A10.

To determine whether the PSA secreted by LNCaP cells is inactivated due to the presence of a protease inhibitor in the conditioned medium, enzymatically active PSA purified from human seminal plasma was added to the flask containing LNCaP cells and conditioned medium. The purified PSA was incubated in this way for 24 h and then concentrated and immunoprecipitated as described previously. Using the HSSKLQ-AMC substrate, it was determined that the PSA isolated in this manner maintained 98.6 \pm 1% of the enzymatic activity of similar concentrations of immunoprecipitated purified PSA. These results demonstrate that the presence of a large amount of inactive PSA in the conditioned medium from LNCaP cells is not due to the presence of an excess of protease inhibitors, nor is it a result of inactivation during the immunoprecipitation process.

Inactivation of the PSA secreted by LNCaP cells could also occur if the majority of the secreted PSA were proteolytically digested by a protease present in the conditioned medium. To confirm whether such a proteolytic inactivation occurs, Western blot analysis of the PSA in the concentrated LNCaP medium was performed (Fig. 2, Lane 6). In this analysis, the isolated PSA is found at the correct M_r 33,000 size as expected for intact PSA, and no significant lower molecular weight bands are detected, indicating that the PSA is not being inactivated secondary to significant proteolysis of the PSA protein.

Ability of PSA from LNCaP Cells to Form Complexes with Protease Inhibitors *in Vitro*. PSA in human serum has been shown previously to be enzymatically inactivated by interaction with the

extracellular protease inhibitors ACT and A2M (19, 20). These inhibitors are present in 10⁴–10⁵-fold molar excess to PSA in serum (19). Enzymatically active PSA forms a 1:1 molar ratio complex with ACT by splitting the reactive center of the inhibitor, thus forming a M_r 90,000 stable PSA-ACT covalent complex, which is enzymatically inactive (19). PSA also forms a complex with the high molecular weight A2M by cleaving a peptide bond in the bait region of A2M (20). This cleavage results in a conformational change in the A2M, and as a result, PSA becomes enveloped within the A2M. It has been demonstrated, however, that PSA can be detected in both the PSA/ACT complex form and the PSA/A2M complex form after SDS/heat denaturing and SDS gel electrophoresis (20).

On the basis of the previous assays using the HSSKLQ-AMC substrate, it appeared that <20% of the PSA secreted by LNCaP cells *in vitro* is enzymatically active PSA. To determine whether a portion of the PSA isolated from LNCaP-conditioned medium is enzymatically inactive because of complex formation with ACT secreted by these cells, direct analysis of the medium by Western blot was performed. As seen in Fig. 2 (Lane 6) PSA in conditioned serum-free medium from LNCaP is found in the M_r 33,000 uncomplexed form. The addition of excess ACT or A2M to the LNCaP-conditioned medium results in the appearance of either the M_r 90,000 band indicative of the PSA/ACT complex or the M_r 180,000 and M_r >300,000 bands indicative of PSA/A2M complexes (Fig. 2, Lanes 7 and 8). Regardless of the inhibitor used, however, most of the PSA is still detectable in the M_r 33,000 uncomplexed form (*i.e.*, >75% using serial dilution standards and densitometric analysis). In contrast, when enzymatically active purified PSA is incubated with these inhibitors, a >90% decrease in the M_r 33,000 band intensity is observed (Fig. 2, Lanes 2 and 3). These results are similar to the findings of the enzymatic assays using the HSSKLQ-AMC substrate and confirm that most of the PSA secreted by LNCaP cells is enzymatically inactive.

Activity of Doxorubicin Prodrugs against LNCaP Cells *in Vitro*. Although the above results demonstrate that LNCaP cells produce a low percentage of enzymatically active PSA, this cell line is the only prostate cancer cell line that continues to secrete PSA during serial passage *in vitro*, and, therefore, it was used to test the cytotoxicity of doxorubicin prodrugs. In initial studies, doxorubicin was coupled directly to the

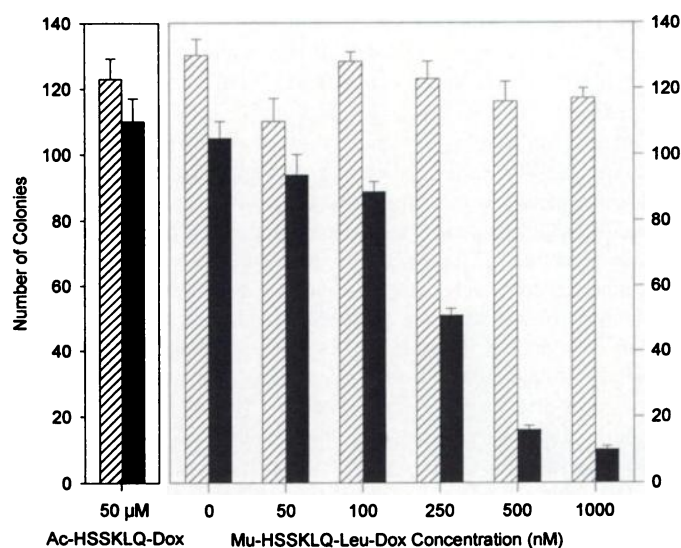


Fig. 4. Clonogenic survival of TSU-Pr1 cells following 48-h treatment with Mu-HSSKLQ-Leu-Dox prodrug with (■) and without (▨) enzymatically active PSA (30 μ g/ml). Results are also shown for HSSKLQ-Dox at a 50 μ M concentration with (■) and without (▨) PSA (30 μ g/ml). Results shown are average ($n = 5$; bars, SE), and assays were done in triplicate.

Ac-HSSKLQ peptide to form the prodrug Ac-HSSKLQ-Dox. Using HPLC detection, it was determined that PSA was unable to hydrolyze the amide bond between the doxorubicin amine and the COOH-terminal glutamine of the peptide (Fig. 3A). In medium containing enzymatically active PSA (30 $\mu\text{g}/\text{ml}$), this prodrug had no demonstrable cytotoxicity at concentrations up to 50 μM (Fig. 4).

A previously described doxorubicin derivative consisting of the amino acid L-leucine linked to the primary amine of doxorubicin (designated Leu-Dox) was demonstrated to have activity against cancer cell lines and has been shown to have less cardiac toxicity than doxorubicin in animal models (21, 22). Leu-Dox was found to have a LD_{50} of 50 ± 8 nm against LNCaP cells. Incubation of the Mu-HSSKLQ-Leu-Dox prodrug with enzymatically active PSA resulted in production of Leu-Dox as determined by HPLC, demonstrating that PSA can hydrolyze the glutamine-leucine peptide bond (Fig. 3B). After 72-h exposure to PSA-producing LNCaP cells, the Mu-HSSKLQ-Leu-Dox prodrug is >90% hydrolyzed to Leu-Dox (Fig. 3C). The Mu-HSSKLQ-Leu-Dox prodrug was then tested against the LNCaP cells and found to be cytotoxic at concentrations as low as 0.1 μM (*i.e.*, $61 \pm 2\%$ inhibition at 0.1 μM and $85 \pm 2\%$ at 1 μM), with a calculated LD_{50} value of 70 ± 5 nm. These results indicate that LNCaP cells secrete sufficient enzymatically active PSA to release proteolytically the cytotoxic moiety Leu-Dox from the initially inactive Mu-HSSKLQ-Leu-Dox prodrug.

Specificity of the Mu-HSSKLQ-Leu-Dox Prodrug. To document that the toxicity of the Mu-HSSKLQ-Leu-Dox prodrug was dependent on PSA-catalyzed hydrolysis, the cytotoxic response of the TSU-Pr1 PSA-nonproducing human prostate cancer cell line was tested. Unlike LNCaP cells, TSU cells can be used in clonogenic survival assays to accurately determine the cytotoxicity of the Mu-HSSKLQ-Leu-Dox prodrug with and without the addition of enzymatically active seminal plasma-purified PSA to the medium. The LD_{50} for Leu-Dox against TSU cells is 120 ± 4 nm. When enzymatically active PSA was not present, a LD_{50} value for Mu-HSSKLQ-Leu-Dox could not be determined at concentrations up to 1 μM (*i.e.*, clonogenic survival at 1 μM dose not statistically different from control). In contrast, when purified, enzymatically active PSA (*i.e.*, 30 $\mu\text{g}/\text{ml}$) was added to serum-containing medium, the LD_{50} was determined to be 230 ± 5 nm.

Conclusion

These studies demonstrate that PC-82 tumors, when grown as primary cultures *in vitro*, secrete high levels of enzymatically active PSA (*i.e.*, 70 ± 5 ng/ 10^6 cells/24 h). In contrast, LNCaP cells secrete low levels of enzymatically active PSA (*i.e.*, 2.3 ± 1 ng/ 10^6 cells/24 h). However, LNCaP cells still secrete sufficient amounts of enzymatically active PSA to activate the Mu-HSSKLQ-Leu-Dox prodrug. The specificity of the cytotoxic response to the Mu-HSSKLQ-Leu-Dox prodrug was documented by the difference in response between the PSA-producing LNCaP cells, in which the LD_{50} value was 70 nm, and PSA nonproducing TSU cells, in which doses as high as 1 μM produce no loss clonogenic ability.

The range of enzymatically active PSA production in the PC-82 and LNCaP cells mimics the situation observed clinically in patients with metastatic prostate cancer. In such patients, metastatic sites are typically composed of prostate cancer cells producing varying amounts of PSA. Previously, it was demonstrated that >90% of the PSA isolated from the extracellular fluid of primary prostate cancers is enzymatically active (11). Once this enzymatically active PSA reaches the blood, it is completely inactivated (11). More than 80% of this inactivation is due to the formation of complexes with ACT and A2M (19, 23). The remaining "free" PSA (*i.e.*, the noncomplexed form) is also not enzymatically active (11). Thus, the PSA-specific prodrug Mu-HSSKLQ-Leu-Dox should not be activated within the circulation. In contrast, once the prodrug diffuses

into the extracellular fluid within sites of prostate cancer metastases, it should be hydrolyzed to the active cytotoxin due to the unique presence of high PSA enzymatic activity. Currently, the Mu-HSSKLQ-Leu-Dox prodrug is being synthesized in sufficient quantities to allow *in vivo* studies in LNCaP and PC-82 tumor-bearing mice.

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