Prostate-Specific Antigen-Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer

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Background: **Standard anti-proliferative chemotherapy is relatively ineffective against slowly proliferating androgen-independent prostate cancer cells within metastatic sites. In contrast, the lipophilic cytotoxin thapsigargin, which causes apoptosis by disrupting intracellular free Ca^{2+} levels, is effective against both proliferative and quiescent (i.e., G_0-arrested) cells. However, thapsigargin's mechanism of action indicates that it is unlikely to be selective for cancer cells or prostate cells.** ***Methods:*** **We coupled a chemically modified form of thapsigargin, L12ADT, to a peptide carrier that is a substrate for the prostate-specific antigen (PSA) protease to produce a soluble, cell-impermeant latent prodrug that is specifically activated extracellularly within metastatic prostate cancer sites by PSA. We analyzed the kinetics of PSA hydrolysis of the prodrug, the *in vitro* cytoxicity of the prodrug against PSA-producing LNCaP human prostate cancer and PSA non-producing HCT-116 human colon cancer cells, and the *in vivo* pharmacokinetics of the prodrug in mice. We also analyzed antitumor efficacy of the prodrug in nude mice xenograft models of prostate cancer (using LNCaP cells) and renal carcinoma (using human SN12C cells).** ***Results:*** **The L12ADT peptide prodrug was hydrolyzed efficiently by PSA, was selectively toxic to PSA-producing prostate cancer cells *in vitro,* and was stable in human plasma. A single dose of 7 mg/kg resulted in a peak serum prodrug concentration of 15.4 ± 1.1 μ*M* and a half-life of approximately 2.8 hours. Over 24 hours, less than 0.5% of free L12ADT was observed in plasma. Levels of prodrug and liberated L12ADT in prostate cancer xenograft tumors were approximately eightfold and sixfold, respectively, higher than the *in vitro* LD_{50}s. Prostate cancer xenograft tumors in mice treated with prodrug by intravenous administration were growth-inhibited without substantial host toxicity. Continuous subcutaneous prodrug administration in mice produced complete growth inhibition of established PSA-producing prostate cancer xenograft tumors but had no effect on PSA non-producing renal carcinoma xenograft tumors.** ***Conclusion:*** **Further development of PSA-activated thapsigargin prodrugs as therapy for metastatic prostate cancer is warranted. [J Natl Cancer Inst 2003;95:990–1000]**

At clinical presentation, primary and metastatic prostate cancers are heterogeneously composed of androgen-dependent and androgen-independent cells *(1).* Following androgen ablation therapy, the androgen-dependent cells undergo apoptosis, resulting in an initial clinical response *(2).* Eventually, all patients relapse and become unresponsive to further anti-androgen therapy, no matter how completely given, because of the presence of androgen-independent prostate cancer cells within the metastatic sites *(3).* Although these cells retain the basic cellular machinery to undergo apoptosis following exposure to a variety of agents, androgen ablation does not induce apoptosis in them because of a defect in the initiation step *(4–8).* Specifically, androgen ablation is unable to induce a sustained elevation in intracellular free Ca^{2+} (Ca_i) levels in these androgen-independent cells. Conversely, agents that increase Ca_i (i.e., calcium ionophores) activate apoptosis in both normal and malignant prostate cells *(5–11).*

These observations provided the rationale for the development of targeted prodrugs that could selectively elevate Ca_i, leading to induction of apoptosis in androgen-independent prostate cancer cells as a new therapy for metastatic prostate cancer *(5,6).* Such a prodrug approach was initiated using thapsigargin (Fig. 1, A). Thapsigargin, purified from the umbelliferous plant *Thapsia garganica (12),* is a potent (active at nanomolar concentrations) inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump *(6,13,14).* Inhibition of the SERCA pump by thapsigargin leads to a rapid, three- to fivefold elevation in Ca_i due to emptying of the stored calcium pools within the endoplasmic reticulum (ER) through the continuous, passive leakage of Ca^{2+} from the ER *(13).* Depletion of the ER Ca^{2+} pool also generates a signal that induces a change in the permeability of the plasma membrane, leading to an influx of Ca^{2+} that is due to the high extracellular Ca^{2+} concentration (1–3 m*M*) *(15).*

By longitudinally monitoring Ca_i within individual cells for up to 7 days *(16),* we demonstrated that continuous exposure to 50–100 n*M* thapsigargin induces a biphasic elevation of Ca_i in prostate cancer cell lines *(8,16,17).* The initial elevation, to 200–400 n*M* Ca^{2+}, occurs within minutes of exposure and lasts for 4–6 hours *(18).* Ca_i levels then return to baseline before a second elevation to micromolar levels, which occurs after 24–72 hours of thapsigargin exposure. This second increase in Ca_i levels is asynchronous within the cell population but ultimately occurs in every dying cell *(8,16,19).* Before the second increase in Ca_i, the expression of a series of Ca^{2+}-regulated proteins (e.g., calmodulin, PAR-4, GADD 153, and GRP-78) increases, even though general protein synthesis decreases *(6,17–19).* The second increase in Ca_i to micromolar levels activates calcineurin, a cal-

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See "Notes" following "References."

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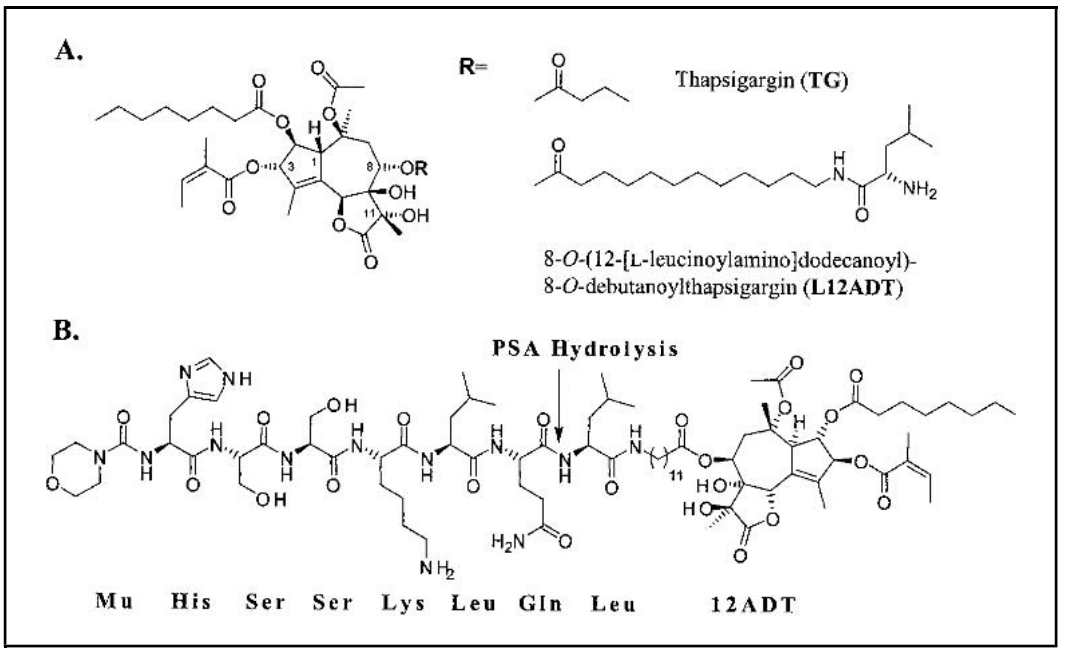

Fig. 1. Chemical structures of thapsigargin and thapsigargin prodrug. **A**) Structures of thapsigargin and L12ADT. **B**) Structure of Mu-HSSKLQ//L12ADT. The **arrow** indicates the site of prostate-specific antigen (PSA) hydrolysis.

cium-dependent phosphatase, which dephosphorylates a series of proteins that includes the pro-apoptotic protein Bad. Dephosphorylated Bad translocates from the cytoplasm to the mitochondria where it can dimerize with either Bcl-2 or Bcl-x_L displacing Bax. The result is a Bax-dependent permeability change in the mitochondrial outer membrane that leads to the release of cytochrome *c* and apoptosis-inducing factor (AIF) *(19,20).* Cytochrome *c* and AIF then activate calpains and caspases, resulting in the morphological changes and genomic DNA fragmentation associated with apoptosis *(8,16,17,21).*

The most important characteristic of thapsigargin is its ability to induce apoptosis in quiescent (i.e., G_0-arrested) as well as proliferating prostate cancer cells *(22).* Thapsigargin's cytotoxicity is, however, unlikely to be prostate- or cell type–specific, given its mechanism of action; therefore, it most likely cannot be administered systemically as a therapeutic agent without substantial host toxicity. In 1993, we described a prodrug approach that was based on coupling a primary amine–containing thapsigargin analog to a peptide carrier to produce a more water soluble compound that could be delivered systemically *(23).* This prodrug is inactive because the carrier peptide prevents it from entering cells until the thapsigargin analog is liberated from the carrier peptide by proteolytic digestion.

Both normal and malignant prostate epithelial cells secrete large amounts of prostate-specific antigen (PSA) *(24–26).* PSA is a serine protease with chymotrypsin-like substrate specificity that is proteolytically active in the extracellular fluid of prostate cancers but inactive in the blood serum, where it forms a complex with the abundant serum protease inhibitors α-1-antichymotrypsin and α-2-macroglobulin *(24,25,26).* A prostate cancer–targeted thapsigargin prodrug could be developed by coupling a primary amine–containing analog of thapsigargin to a PSA-specific peptide carrier via a PSA-cleavable peptide bond *(23,27).* To accomplish this, we identified a highly selective and efficient peptide substrate for PSA with the sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) *(28).* A fluorescent peptide substrate was produced by coupling the HSSKLQ peptide to 7-amino-4-methyl coumarin. Using this substrate, we confirmed that the extracellular fluid surrounding prostate cancers contained high concentrations of enzymatically active PSA that could hydrolyze this substrate *(29).* In contrast, this substrate was stable in plasma, even from patients with high plasma PSA levels *(29).*

The goal of the present study was to synthesize and characterize a prodrug consisting of a primary amine–containing thapsigargin analog coupled to the PSA-specific peptide HSSKLQ to form a PSA-cleavable peptide bond. We previously described thapsigargin analogs in which the *O*-8 butanoyl group was substituted with various primary amine–containing side chains and characterized their ability to inhibit the SERCA pump, to elevate Ca_i in intact whole prostate cancer cells, and to exhibit cytotoxicity against human prostate cancer cell lines *(30,31).* Initial attempts to directly couple primary amine–containing toxins to the C-terminal carboxyl group of glutamine resulted in prodrugs that were hydrolyzed inefficiently by PSA *(32).* Subsequently, however, we demonstrated that PSA could hydrolyze the toxin–peptide conjugates when the amino acid leucine was introduced as a linker between the peptide and the primary amine–containing toxin *(32,33).* On the basis of this observation, we coupled a 12-aminododecanoyl side chain analog of thapsigargin (12ADT) to leucine to produce L12ADT (Fig. 1, A), an analog that is as potent a cytotoxin as thapsigargin (i.e., the dose that is lethal to 50% of the cells [LD_{50}] is approximately 30 n*M*) *(31).* In this article, we further characterize the cytotoxic properties of thapsigargin and L12ADT. We then describe the analysis of the kinetics of PSA hydrolysis and cytotoxicity of the prodrug *in vitro.* We also report the *in vivo* pharmacokinetics of the prodrug and its antitumor efficacy and host toxicity in nude mice xenograft models.

Materials and Methods

Cell Lines and Culture Conditions

Cell lines were maintained by serial passage in the indicated medium in 5% CO_2/95% air at 37 °C. The LNCaP, DU145, and

PC-3 human prostate cancer cell lines (RPMI-1640, 10% fetal bovine serum [FBS]), the HCT-116 human colon cancer cell line (McCoy's 5a, 10% FBS), the TT human medullary thyroid cancer cell line (RPMI-1640, 15% FBS), the MCF-7 human breast cancer cell line (Dulbecco's modified Eagle medium [DMEM], 10% FBS), and WI-38 normal human fibroblasts (minimal essential medium [MEM], 10% FBS) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The C4-2B human prostate cancer cells (RPMI-1640, 10% FBS) were from UroCor (Los Angeles, CA), the MDA-PCA-2B human prostate cancer cells (HPC-1 medium, 20% FBS) were from Dr. Nora Navone (The University of Texas M. D. Anderson Cancer Center, Houston, TX), the LAPC-4 human prostate cancer cells (Iscove's medium, 10% FBS, and R1881 at 1 n*M*) were from Dr. Charles Sawyer (University of California at Los Angeles), the SN12C human renal cancer cells (Eagle's mimimum essential medium [EMEM], 10% FBS) were from Dr. Isaiah Fidler (The University of Texas M. D. Anderson Cancer Center), the CWR22R human prostate cancer cells (RPMI-1640, 10% FBS) were from Dr. J. Jacobsen (Case Western Reserve University, Cleveland, OH), and the TSU human bladder cancer cells (RPMI-1640, 10% FBS) were from Dr. T. Itzumi (Teikyo University, Tokyo, Japan). Normal human osteoblasts (NHO; OGM-defined medium), normal human macrovein endothelial cells (HuVecs; EGM-2-defined medium), normal human microvein endothelial cells (HMVecs; EGM-2 medium), and normal human prostate epithelial cells (PRECs; PREGM-defined medium) were from Clonetics/Cambrex Bio Science, Walkersville, MD). The OGM-, EGM-2-, PREGM-defined media, and FBS were from Clonetics. RPMI-1640, DMEM, EMEM, MEM, McCoy's 5a, and Iscove's media were from Gibco Life Technologies, Gaithersburg, MD. HPC-1 medium was from Athena Environmental Sciences (Baltimore, MD).

Low-proliferation cultures of LNCaP cells were established according to the method of Pinski et al. *(22)* with the following modifications. The conditionally immortalized human fetal osteoblastic cell line hFOB (obtained from ATCC) was maintained in a 1 : 1 mixture of phenol-free DMEM/Ham's F12 medium containing 10% FBS supplemented with geneticin (300 μg/mL) at 34 °C, the permissive temperature for the expression of the large T antigen. hFOB osteoblasts (10^5 cells) were grown in DMEM/Ham's F12 medium at permissive temperature for 3 days until they were 90%–100% confluent. Confluent cultures of hFOB cells were then growth-arrested by shifting the temperature to 39 °C; they were maintained in the growth-arrested state for 2 additional days. The osteoblast-conditioned medium was then removed and added to LNCaP cells (1×10^5) in a 1 : 1 mixture with RPMI-1640, and the cells were incubated for 3 days as monocultures. LNCaP cells (1×10^5) were then incubated for 3 days at 37.5 °C in a 1 : 1 mixture of osteoblast-conditioned medium and RPMI-1640 medium as a monoculture. Chemotherapeutic agents (i.e., doxorubicin, paclitaxel [Taxol; Sigma-Aldrich, St. Louis, MO], or thapsigargin) were then added to the unchanged medium at concentrations of 10, 100, or 1000 n*M*. After 48 hours, the cells were treated with 0.05% trypsin in Hank's balanced salt solution (HBSS; Gibco), washed with sterile saline, fixed in methanol, and incubated with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/μL (Sigma) in saline to stain nuclear DNA. The percentage of apoptotic nuclei was determined by evaluating nuclear morphology with epifluorescence microscopy.

Materials

PSA was purified from human seminal plasma, as described *(28)*. All other reagents, unless otherwise specified, were obtained from Sigma-Aldrich. Thapsigargin was ethanol-extracted from the harvested seeds of *T. garganica,* and the thapsigargin analog 8-*O*-(12[L-leucinoylamino]dodecanoyl)-8-*O*-debutanoyl thapsigargin (L12ADT) was synthesized from thapsigargin, as previously described *(31)*.

Mu-HSSKLQ//L12ADT Prodrug Synthesis

Mu-HSSKLQ//L12ADT (where // denotes the PSA cleavage site) was produced by coupling L12ADT to the Mu-HSSK(Fmoc)LQ peptide (where Mu denotes the 4-morpholinecarbonyl amino-terminal protecting group) (California Peptide, Napa, CA). Before use, the peptide was purified by high-pressure liquid chromatography (HPLC), and molecular weight was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and amino acid analysis. Equimolar L12ADT and Mu-HSSK(Fmoc)LQ peptide were coupled at room temperature by using a threefold molar excess of 1-hydroxybenzotriazole (HOBT) and 2-diisopropylcarbodiimide (DIC) in dimethylformamide (DMF). The reaction was complete after approximately 6–10 hours. The final product was Fmoc-deprotected in piperidine/DMF and purified by HPLC; the correct mass was confirmed by mass spectrometry.

Kinetic Analysis of PSA Hydrolysis

Various concentrations of Mu-HSSKLQ//L12ADT were incubated with PSA (10 μg/mL final concentration) in 50 m*M* Tris and 0.1 *M* NaCl (pH 7.8) at room temperature. At discrete time points (0.5, 1, 2, 3, and 4 hours), aliquots of the reaction mixture were removed and analyzed by HPLC on a reverse-phase C18 Ultrasphere analytical column (15 cm × 4.6 mm [inner diameter]; Beckman Coulter, Fullerton, CA). An isocratic elution was performed (9% acetonitrile, 0.1% trifluoroacetic acid, H_2O) to visualize the free peptide peak. A standard curve produced by using purified free Mu-HSSKLQ peptide was used to convert peak area to free peptide concentration. Peak areas of free peptide at each time point were then converted to concentration, and the concentration data were analyzed by Lineweaver–Burke plots ($1/V$ versus $1/S$, 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 used to compare hydrolysis of the prodrug with hydrolysis of the fluorescent PSA substrate Mu-HSSKLQ-AMC (where AMC is 7-amino-4-methyl coumarin).

Calcium Measurements

TSU cells were loaded for 30 minutes at room temperature with 7.5 μ*M* fura-2AM (Molecular Probes, Eugene, OR). These cells were then treated with Mu-HSSKLQ//L12ADT prodrug or free L12ADT at various concentrations. Intracellular free calcium levels in treated TSU cells were determined in a cuvette assay, as previously described *(16)*.

Cytochemical Staining

Intracellular caspase 3 activation and fluorescein isothiocyanate (FITC)–annexin V staining were analyzed by using flow cytometry, as previously described *(34)*. The PhiPhiLux-G_1D_2

caspase 3 substrate was obtained from OncoImmunin (Gaithersburg, MD). The Annexin V-FITC apoptosis kit was obtained from BD Pharmingen (San Diego, CA). For AIF immunocytochemical staining, cells were cytospun onto lysine-coated slides, and standard 3,3′-diaminobenzidine-tetrahydrochloride (DAB) immunoperoxidase staining was performed by using polyclonal anti-AIF serum from Santa Cruz Biotechnology (Santa Cruz, CA).

Cytotoxicity Assays

The induction of apoptosis in LNCaP cells after 48 hours of exposure to 100 n*M* of doxorubicin (Sigma-Aldrich), paclitaxel (Sigma-Aldrich), or thapsigargin in standard high-proliferation or low-proliferation culture was assessed by nuclear DAPI staining, as described *(22)*. High versus low proliferation in these cultures was determined on the basis of Ki-67 staining, as described *(22)*. Viability of normal and malignant cell types following 5 days of continuous exposure to thapsigargin at 100 n*M* was determined by using the Promega Cell Titer 96 Non-Radioactive Cell Proliferation Assays (Promega, Madison, WI), according to the manufacturer's instructions *(35)*.

Cytotoxic response to continuous exposure of LNCaP and HCT-116 cells and episodic exposure of LNCaP cells to Mu-HSSKLQ//L12ADT were determined using the Promega Cell Titer Proliferation Assays, as above. For episodic treatments, LNCaP cells were exposed to prodrug in serum-containing medium for 8 hours; the medium was then removed and replaced with fresh serum-containing medium. Medium was changed daily for the duration of the experiment. For continuous exposure experiments, medium containing either L12ADT or Mu-HSSKLQ//L12ADT was not changed over the time course of the experiments. Cell titer proliferation assays were completed at the same time each day, according to the manufacturer's instructions. Standard curves were generated for each cell line to convert absorbance units to cell numbers.

Clinical Samples

Human prostate cancer samples were obtained from the archival collection of the Johns Hopkins School of Medicine Department of Pathology and the warm autopsy program of the National Cancer Institute (NCI)–Specialized Programs of Research Excellence (SPOREs) at Johns Hopkins University. Tissue was procured according to protocols approved by the Johns Hopkins Institutional Review Board. The tissue samples were stained with Ki-67 to determine the growth fraction, as previously described *(22,36)*.

Intratumoral Injection of LNCaP Xenograft Tumors With L12ADT

LNCaP cells (2×10^6) in 100 μL of Matrigel (Collaborative Research, Bethesda, MD) were inoculated into the flank of 6-week-old male nude mice (Harlan Sprague-Dawley, Indianapolis, IN). Tumors were measured with calipers. After approximately 4 weeks, tumors reached 0.5–1 cm^3 and were injected daily with 100 μL of either sterile saline or L12ADT (100 nmol/injection) for two 5-day courses separated by 2 off-days. After the second injection course (i.e., after 12 days), mice were killed by CO_2 overdose, and tumors were harvested, fixed, stained with hematoxylin and eosin, and examined at ×25 magnification with a digital camera. The total area of the tissue section and the area of viable tumor were determined with the ImagePro image analysis package (Media Cybernetics, Carlsbad, CA), as described previously *(37)*.

Determination of Plasma Levels of Mu-HSSKLQ//L12ADT Prodrug

Calibration standards consisted of Mu-HSSKLQ//L12ADT prodrug or L12ADT spiked into mouse plasma, and plasma samples from treated mice were analyzed by liquid chromatography coupled to a quadrupole mass spectrometer (LC/MS/MS; PESciex API 3000; PerkinElmer, Shelton, CT). A multistep gradient elution HPLC method was used to separate the Mu-HSSKLQ//L12ADT prodrug from the free L12ADT with eluent A (2 m*M* ammonium acetate with 0.1% formic acid) and eluent B (90% acetonitrile/10% deionized water) and a gradient of 1005 A to 1005 B over 12 minutes. The system was calibrated with extracted standards of Mu-HSSKLQ//L12ADT or L12ADT at concentrations of 0.001 to 100 μ*M*, and linear regression analysis was used to generate best-fit lines, from which peak areas of samples were converted to concentration of prodrug or L12ADT. Single-dose pharmacokinetics were assessed by noncompartmental analysis *(38)*. The area under the curve from time zero to infinity ($AUC_{0\text{-}\infty}$) was calculated with the linear trapezoidal method *(38)*. The terminal half-life ($t_{1/2}$) was determined from the terminal slope (k_e) on a log-linear plot of concentration versus time.

Determination of Tumor Tissue Levels of Mu-HSSKLQ//L12ADT Prodrug and Free L12ADT

A tumor tissue calibration curve was first constructed by adding an internal standard (8-*O*-[12-{L-serinoylamino}dodecanoyl]-8-*O*-debutanoylthapsigargin [S12ADT]) *(31)* (final concentration ranged from 0.014 to 30 μ*M*) to tumor tissue homogenate obtained from untreated animals. Mice bearing LNCaP tumors, prepared as described above, received an intravenous injection of 7 mg/kg of Mu-HSSKLQ//L12ADT every day for 3 days. One hour after the last injection, the tumors were harvested and homogenized on ice in protease inhibitor (Complete Inhibitor; Boehringer Mannheim, Mannheim, Germany) containing phosphate-buffered saline by using a mechanical tissue grinder. LC/MS (Agilent Technologies, Wilmington, DE) analysis was performed on tumor tissue calibration standards and tumor tissue obtained from treated animals. The mobile phase consisted of 0.1% formic acid in an acetonitrile gradient from 5% to 100% over 16 minutes with a flow rate of 0.5 mL/min. Each compound was discriminated on the basis of the individual extracted ion chromatogram, and the areas of the Mu-HSSKLQ//L12ADT and L12ADT were converted into a ratio with the internal standard.

In Vivo Toxicity Assays

To determine *in vivo* toxicity of thapsigargin and L12ADT, BALB/c mice (Harlan Sprague-Dawley) received a single intravenous injection of an increasing dose of each agent. Mice were monitored for toxicity hourly for the first 12 hours and then daily for 1 week. Separate groups of three mice each received increasing doses of thapsigargin (0.1, 0.2, 0.4, and 0.8 mg/kg) and L12ADT (0.1, 0.2, 0.4, 0.8, and 1.6 mg/kg). Dose escalation was stopped at the dose level that resulted in death of all mice after 1 hour (LD_{100}). All animals receiving doses below the LD_{100} were alive and well up to 1 week after receiving a single dose.

Systemic Efficacy Studies

Male nude mice were inoculated with tumor cells (LNCaP or SN12C), as described above for LNCaP cells. When tumors reached 0.1–0.2 cm^3 (after approximately 2–3 weeks), the mice were separated into two groups such that the average starting tumor volumes were equivalent in the two groups. Tumors were measured with calipers, and mice were weighed twice a week while on treatment. At the end of the experiments, mice were killed by CO_2 overdose, and tumors were harvested and weighed. For intravenous experiments, tumor-bearing mice were administered a daily dose of 7 mg/kg of Mu-HSSKLQ//L12ADT (in 2% dimethyl sulfoxide [DMSO] in H_2O) via tail vein injection. Mice were treated once a day, 5 days a week, for three treatment cycles on days 1–5, 14–18, and 35–39. Control mice were treated similarly but with vehicle only (in 2% DMSO in H_2O). For continuous infusion experiments, osmotic minipumps (Alzet Osmotic Pumps, Cupertino, CA) containing Mu-HSSKLQ//L12ADT (12.4 mg/mL in sterile H_2O containing 10% DMSO) were inserted subcutaneously under sterile conditions through a flank incision that was closed with staples. The osmotic pump delivered the drug at a rate of 0.5 μL/hour for approximately 14 days, for an average daily dose of 7 mg/kg. For 28-day treatment, mice had a second pump inserted after removal of the first drug-depleted pump. Vehicle controls were similarly treated with an osmotic pump containing only 10% DMSO in H_2O. All animal studies were performed according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

Statistical Methods

For the longitudinal comparisons of tumor size (*see* Fig. 6), random-effects linear regression models were estimated using a maximum likelihood approach (Stata 7.0; Stata Corporation, College Station, TX). Outcome data (i.e., relative tumor volume as compared with that on day 0) in the systemic efficacy experiments on LNCaP xenograft tumors were first transformed to the natural log scale to adhere to the assumptions of linear regression (such transformation was not necessary for the outcome data for the SN12C xenograft tumors). Models included covariates for time (in days), treatment status (i.e., control versus treatment), and an interaction between treatment and time. For the continuous infusion experiments with LNCaP xenografts, a quadratic term for time and the interaction between the quadratic of time and treatment were also included in the model to better describe the relationship between time and relative tumor volume (it was not necessary to do so for the SN12C groups). Fitted regression lines for the LNCaP tumors were then back-transformed to the original data scale for graphical display. Log-likelihood statistics were obtained and compared with those from analogous models without treatment-related covariates using chi-square statistics. A *P* value comparing models with and without treatment was calculated using the chi-square statistic. All statistical tests were two-sided.

RESULTS

Advantage of Thapsigargin in Treatment of Prostate Cancer

A variety of agents are able to effectively induce apoptosis of human prostate cancer cells in standard *in vitro* assays. In such standard culture conditions, the growth fraction of the human prostate cancer cell lines during drug exposure is characteristically greater than 90% (Fig. 2, A). In contrast, the growth fraction of malignant cells within metastatic tissue obtained at autopsy from patients who had failed androgen ablative therapy is less than 10% (Fig. 2, A). To compare the sensitivity of androgen-independent prostate cancer cells to thapsigargin with their sensitivity to antiproliferative chemotherapy agents, we used an assay system that we developed that more closely mimics the clinical situation. In this assay system, LNCaP human prostate cancer cells are shifted into a low–growth fraction state by culturing in medium conditioned by non-proliferating human os-

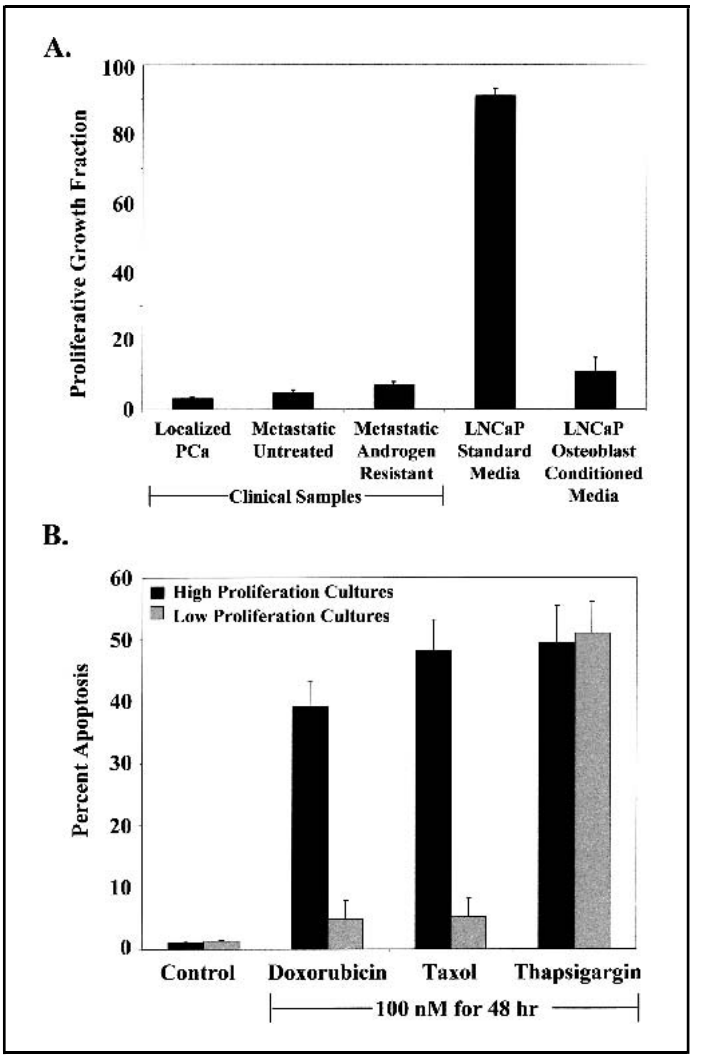

Fig. 2. Growth of human prostate cancer cells and effect of thapsigargin. **A**) Proliferative growth fraction of human prostate cancer samples and of LNCaP human prostate cancer cells growing in standard medium or in medium conditioned by human osteoblasts. Clinical samples include localized PCa (prostate cancers obtained from radical prostatectomy specimens [n = 27]); metastatic untreated (prostate cancers obtained from men who had not received androgen-ablative therapy [n = 43]); and metastatic androgen resistant (prostate cancers obtained at warm autopsy from men who had failed androgen-ablative therapy [n = 132]). **Error bars** indicate upper 95% confidence intervals. **B**) Percentage of LNCaP cells undergoing apoptosis after treatment with vehicle or 100 n*M* doxorubicin, paclitaxel, or thapsigargin in standard medium (high-proliferation cultures) or in osteoblast-conditioned medium (low-proliferation cultures). Cells were exposed to drug for 48 hours and were then fixed and stained with DAPI to assess nuclear morphology. Percentage apoptosis indicates percentage of counted cells (n = 200 for each drug) with apoptotic nuclear morphology.

teoblasts *(22).* Using this system, the growth fraction can be shifted from approximately 90% to 10%–20% without loss of cell viability (i.e., >95% viable cells at 1 week of such low–growth fraction culture) (Fig. 2, A). The induction of apoptosis in LNCaP cells treated with paclitaxel, doxorubicin, or thapsigargin was examined in standard high-proliferation and low-proliferation culture conditions (Fig. 2, B). Whereas all of these agents were equally effective at inducing apoptosis of cells in the high–growth fraction cultures, only thapsigargin retained its effectiveness when assayed in the low-proliferation cultures (Fig. 2, B).

Because it inhibits the SERCA pump, which is critical for maintaining intracellular calcium homeostasis in all cell types, thapsigargin's cytotoxicity is unlikely to be malignancy- or cell type–specific. To examine the generality of the potent cytotoxic effects of thapsigargin, a series of normal and malignant cells were exposed to 100 n*M* thapsigargin for 5 days. Such exposure resulted in an 80%–90% reduction in viable cell number, regardless of whether the cells were normal or malignant or of prostatic origin (Table 1). This cytotoxic response was not limited to a particular subtype of prostate cancer and occurred regardless of the androgen receptor status or responsiveness of the cell (Table 1). These observations confirmed our supposition that, although thapsigargin could be an effective drug for prostate cancer therapy, it cannot be administered systemically without significant host toxicity. To confirm that systemic administration would in fact be toxic, mice were given increasing doses of thapsigargin intravenously (0.1, 0.2, 0.4, and 0.8 mg/kg). At a thapsigargin dose of 0.8 mg/kg (1280 nmol/kg), all mice (n = 5) died within 1 hour. These studies document the systemic toxicity of and affirm the need for targeted delivery of thapsigargin analogs by the prodrug approach.

Targeting Thapsigargin to Prostate Cancer Cells

Because there is no inherent therapeutic index for thapsigargin, a strategy is required for targeting thapsigargin to prostate cancer sites to prevent toxicity to normal cells. We used L12ADT, which we had identified as having cytotoxic potency identical to that of thapsigargin *(31).* We first investigated the mechanism of cytotoxicity of L12ADT. Like thapsigargin, L12ADT rapidly entered TSU cells and produced an initial elevation of Ca_i (Fig. 3, A), presumably due to inhibition of the SERCA pump. The initial elevation of Ca_i, to 200–400 n*M,* occurred within minutes, and Ca_i then returned to baseline (i.e., 20–40 n*M*) within 6–8 hours. A second sustained elevation to above 10 μ*M* Ca_i occurred between 18 and 96 hours of continuous drug exposure (data not shown). The second Ca_i elevation was associated with the translocation of cytochrome *c* from the cytoplasm to the mitochondria and translocation of AIF from the mitochondria to the nucleus (Fig. 3, B). These changes induced the apoptotic cascade, resulting in activation of caspase 3 (Fig. 3, C) and externalization of phosphotidylserine to the extracellular surface of the plasma membrane, as visualized by annexin V staining (Fig. 3, D). Eventually the cells underwent plasma membrane blebbing and subsequent fragmentation into apoptotic bodies (Fig. 3, E and F).

Previously, we had reported that the LD_{50} of L12ADT for human TSU bladder cancer cells was 30 ± 10 n*M (31).* The LD_{50} of L12ADT for LNCaP cells following 72 hours of continuous exposure was 13 ± 3 n*M.* To determine whether L12ADT has a similar cytotoxic effect *in vivo,* nude mice (n = 6 per group) bearing LNCaP tumors received two 5-day courses, separated by 2 off-days, of intratumoral inoculations of either vehicle or L12ADT (approximately 100 nmol/injection, producing a theoretical intratumoral concentration of 100–200 n*M*). No ill effects were observed in the host mice (data not shown). The volume of viable tumor cells in the vehicle control group (n = 6) at the end of 12 days was 152% (95% CI = 72% to 232%) of the starting volume, whereas in the L12ADT-treated mice (n = 6), the volume was 45% (95% = 15% to 75%) of the starting volume. The difference in the volume change was statistically significant ($P = .04$), indicating that L12ADT had the potential to be an effective therapy for prostate cancer without host toxicity if sufficient concentrations (i.e., 100–200 n*M*) of L12ADT could be targeted specifically to tumor sites. However, as expected, L12ADT, like thapsigargin, also possessed substantial systemic toxicity *in vivo* when administered intravenously to non–tumor-bearing BALB/c mice (i.e., a dose of 1.6 mg/kg [2560 nmol/kg] killed 100% of the mice [n = 5] within 1 hour of intravenous dosing).

Table 1. Cytotoxic responses of normal and malignant cell lines to thapsigargin

Human cell type	Designation	Androgen receptor (gene status)	Androgen growth responsiveness	Cellular response to thapsigargin (% decrease in viable cells)*
Prostate cancer	LNCaP	+ (mutant)	+	88 (76 to 100)
Prostate cancer	MDA-PCA-2B	+ (mutant)	+	79 (69 to 89)
Prostate cancer	LAPC-4	+ (wild type)	+	85 (81 to 89)
Prostate cancer	C4-2B	+ (mutant)	–	91 (83 to 99)
Prostate cancer	CWR22R	+ (mutant)	–	89 (79 to 99)
Prostate cancer	DU-145	–	–	78 (66 to 90)
Prostate cancer	PC-3	–	–	88 (82 to 94)
Renal cancer	SN12C	–	–	82 (70 to 94)
Bladder cancer	TSU	–	–	91 (83 to 99)
Colon cancer	HCT-116	–	–	90 (78 to 102)
Medullary thyroid cancer	TT	–	–	82 (68 to 96)
Breast cancer	MCF-7	+ (wild type)	–	83 (79 to 87)
Normal fibroblast	WI-38	–	–	79 (67 to 91)
Normal osteoblast	NHO	+ (wild type)	–	81 (67 to 95)
Normal microvein endothelial cells	HMVEC	–	–	85 (73 to 97)
Normal macrovein endothelial cells	HuVec	–	–	82 (78 to 86)
Normal prostate epithelial cells	PREC	–	–	91 (83 to 99)

*Decrease in number of viable cells after 5-day treatment with 100 n*M* thapsigargin, expressed as a percentage of cells treated with vehicle only. Data are given as means and 95% confidence intervals.

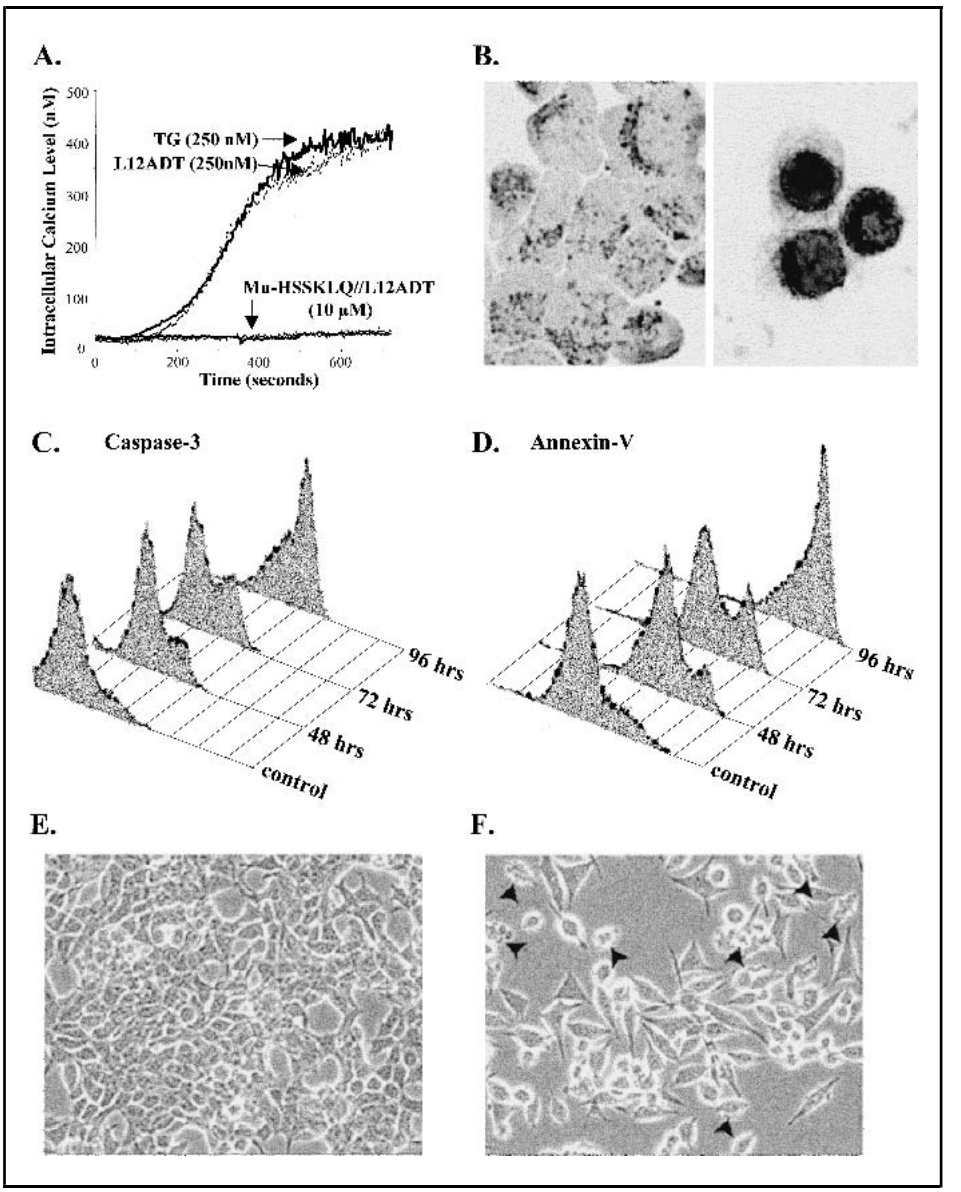

Fig. 3. Analysis of *in vitro* response of cancer cells to free L12ADT or Mu-HSSKLQ//L12ADT prodrug. **A**) Fura-2 ratiometric analysis of intracellular free Ca^{2+} response in TSU human bladder cancer cells (prostate-specific antigen [PSA] nonproducing) exposed to either 250 n*M* thapsigargin (TG), 250 n*M* L12ADT, or 10 μ*M* Mu-HSSKLQ//L12ADT prodrug. **B**) Immunohistochemical analysis of translocation of apoptosis-inducing factor from mitochondria in vehicle-treated LNCaP human prostate cancer cells (**left panel**) to nuclei of cells treated for 48 hours with 200 n*M* L12ADT (**right panel**). **C**) Flow cytometric analysis of the increase in the proportion of unfixed LNCaP cells positive for enzymatically active caspase 3 after exposure to 200 n*M* L12ADT. **D**) Flow cytometric analysis of the increase in proportion of unfixed LNCaP cells positive for fluorescein isothiocyanate (FITC)–annexin V staining of the plasma membrane following exposure to 200 n*M* L12ADT. **E**) Phase contrast microscopy analysis of unfixed vehicle-treated LNCaP cells. **F**) Phase contrast microscopy analysis of unfixed LNCaP cells after 48 hours of exposure to 200 n*M* L12ADT. **Arrowheads** denote several cells undergoing membrane blebbing as part of their apoptotic death (magnification = ×320).

In Vitro Characterization of Mu-HSSKLQ//L12ADT Prodrug

Because of its systemic toxicity, we coupled L12ADT to the Mu-HSSKLQ peptide carrier to produce the prodrug Mu-HSSKLQ//L12ADT (Fig. 1, B). Such peptide coupling should prevent L12ADT from entering cells and inhibiting the SERCA pumps, thereby rendering it nontoxic. To determine whether this prevention occurs, PSA non-producing TSU bladder cancer cells were exposed to Mu-HSSKLQ//L12ADT at 10 μ*M* (Fig. 3, A) At this dose level, no appreciable increase in Ca_i was detected (Fig. 3, A). In contrast, a 40-fold lower concentration of L12ADT produced maximal elevation in Ca_i (Fig. 3, A). These results suggest that the addition of the Mu-HSSKLQ peptide does prevent the L12ADT analog from entering cells. *In vitro,* PSA cleaved L12ADT from the prodrug peptide with a K_m of 475 μ*M,* a k_{cat} of 0.0096 s^{-1}, and a k_{cat}/K_m of 21.9 $s^{-1}M^{-1}$. These kinetics were similar to those previously reported for the fluorescent substrate Mu-HSSKLQ//AMC [i.e., K_m = 470 μ*M,* k_{cat} = 0.011, k_{cat}/K_m = 23.6 $s^{-1}M^{-1}$ *(28)*]. In 5-day *in vitro* exposure cytotoxicity assays, the LD_{50} of the Mu-HSSKLQ//L12ADT prodrug against PSA-producing LNCaP human prostate cancer cells was 74 ± 3 n*M.*

Continuous exposure of cells *in vitro* does not mimic the expected episodic exposure to any drug administered systemically daily *in vivo.* This problem is particularly relevant with regard to a cytotoxic agent such as L12ADT which, because of its high lipophilicity, is concentrated by cells from the aqueous environment and retained after it is liberated from the prodrug. Therefore, daily exposure of cells to cytotoxic agents for short periods of time may represent an *in vitro* model that is more relevant to the drug exposure conditions observed *in vivo.* Thus, the response of LNCaP cells to episodically administered prodrug (i.e., 8 h/day) was compared with the response to prodrug given continuously (Fig. 4, A). Continuous exposure of LNCaP cells to 500 n*M* prodrug resulted in nearly 2 logs of cell kill by day 7. Similar results were obtained with episodic exposure to 1000 n*M* prodrug (Fig. 4. A). A more delayed cytotoxic effect was observed following episodic exposure to 500 n*M* prodrug (Fig. 4, A). As a control for the specificity of this response, PSA non-producing HCT-116 cells were exposed continuously to 500 n*M* prodrug. In contrast to the response of PSA-producing

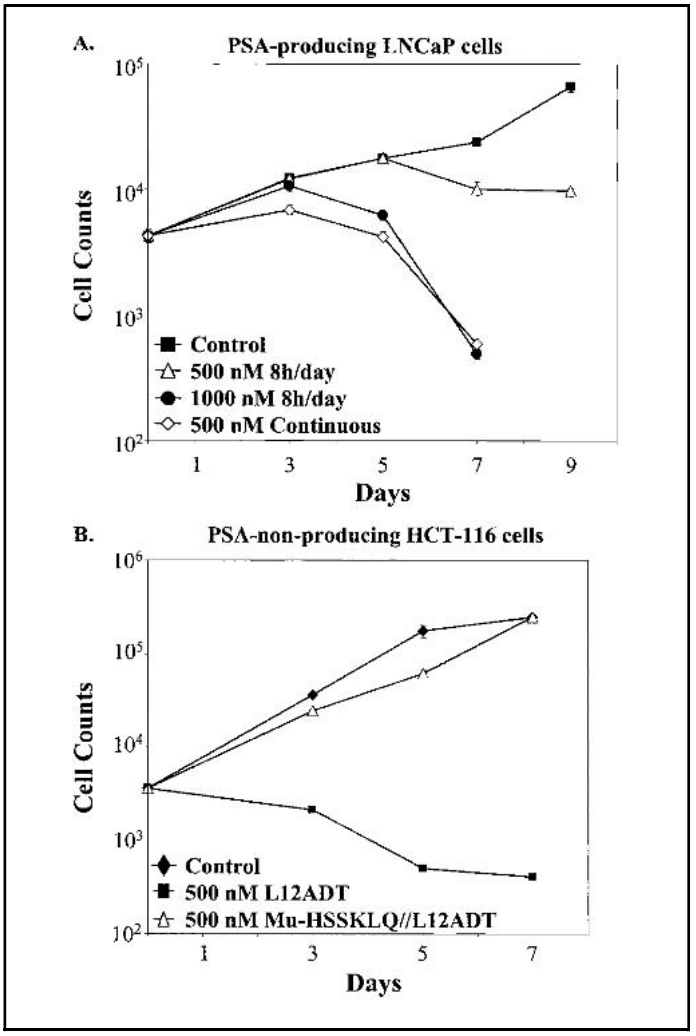

Fig. 4. Cytotoxic response of cancer cells to continuous and episodic administration of L12ADT and Mu-HSSKLQ//L12ADT prodrug. **A**) Cell viability of prostate-specific antigen (PSA)-producing LNCaP cells treated continuously with 500 n*M* Mu-HSSKLQ//L12ADT, episodically with 500 or 1000 n*M* Mu-HSSKLQ//L12ADT, or with vehicle. For episodic treatments, cells were exposed to prodrug in serum-containing medium for 8 hours; the medium was then removed and replaced with fresh serum-containing medium. Medium was then changed daily. **B**) Cell viability of PSA non-producing HCT-116 human colon cancer cells treated continuously with 500 n*M* L12ADT or Mu-HSSKLQ// L12ADT. Cell viability assays were completed on the indicated days. Standard curves were generated for each cell line to convert absorbance units to cell numbers. Each data point represents the average of data from eight wells from one experiment. **Error bars** indicate 95% confidence intervals. Experiments were duplicated and yielded similar results (data not shown).

LNCaP cells, no substantial effect on cell viability was observed, even though these cells were highly sensitive (i.e., approximately 2 logs of cell kill by day 5) to 500 n*M* free L12ADT (Fig. 4, B).

Pharmacokinetic Studies of Mu-HSSKLQ//L12ADT Prodrug

Levels of prodrug and L12ADT in the blood of mice (n = 3 for each of seven time points) were measured by LC-MS. The C_{max} of the prodrug 5 minutes after a single intravenous injection of 7 mg/kg was 15.4 ± 1.1 μ*M,* and the $t_{1/2}$ was 2.8 ± 0.02 hours (Fig. 5). In contrast, the C_{max} for L12ADT was observed at 12 hours after prodrug injection and was only 10.3 ± 2 n*M*

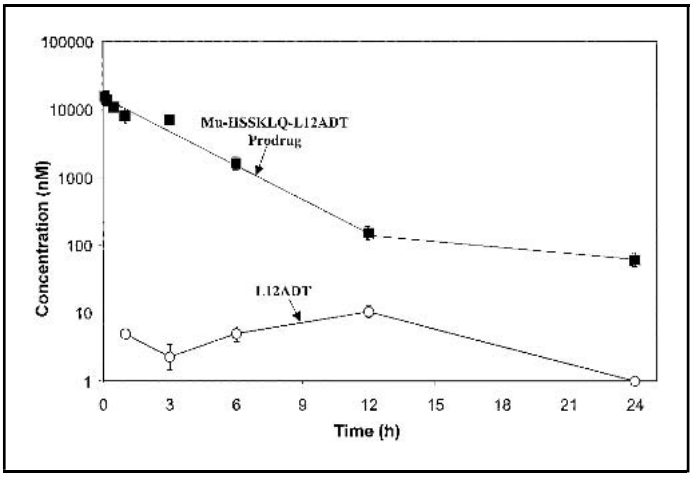

Fig. 5. Pharmacokinetic analysis of plasma levels of Mu-HSSKLQ//L12ADT and L12ADT. Male mice were given a single intravenous injection of prodrug at 7 mg/kg. At the indicated time points, blood was obtained from groups of three anesthetized mice, and the plasma was isolated and extracted. Serum samples spiked with either Mu-HSSKLQ//L12ADT or L12ADT underwent a similar extraction procedure and were used as calibration standards. Data represent averages, with **error bars** representing 95% confidence intervals for groups of three mice at each time point. For Mu-HSSKLQ//L12ADT C_{max} = 15.4 μ*M* (95% confidence interval [CI] = 13.3 to 17.5 μ*M*) at 5 minutes post injection, $t_{1/2}$ = 2.8 hours (95% CI = 2.78 to 2.82 hours), and area under the curve (AUC) = 2538 μ*M* min. Very low levels of L12ADT were detected in serum of mice injected with Mu-HSSKLQ-L12ADT, with a C_{max} of 0.010 ± 0.002 μ*M* at 12 hours and AUC of 8.1 μ*M* min. The ratio of the AUC for L12ADT to the AUC for Mu-HSSKLQ//L12ADT was 0.3 ± 0.06%.

(Fig. 5). The Mu-HSSKLQ//L12ADT prodrug was stable in blood, with less than 0.5% of the prodrug non-specifically hydrolyzed to the free L12ADT over a 24-hour period (i.e., the ratio of the AUC for the prodrug to AUC for L12ADT was 0.3 ± 0.03%).

The data in Fig. 5 demonstrate that blood levels of Mu-HSSKLQ//L12ADT above the *in vitro* LD_{50} for LNCaP cells (approximately 75 n*M*) were sustained for approximately 12 hours following a 7-mg/kg intravenous injection. To determine the intratumoral levels of both prodrug and free L12ADT, mice bearing LNCaP xenografts (n = 4) were injected intravenously for 3 consecutive days with the prodrug at 7 mg/kg, and tumors were harvested 1 hour after the third dose. The intratumoral concentration of Mu-HSSKLQ//L12ADT was 640 ± 80 n*M* (i.e., 8.5 times the LNCaP LD_{50} for Mu-HSSKLQ//L12ADT *in vitro*) and 170 ± 58 n*M* for the liberated L12ADT (i.e., 13 times the LNCaP LD_{50} for L12ADT *in vitro*). These results demonstrate that a dose of 7 mg/kg of prodrug can produce effective tumor tissue levels that are cytotoxic to cancer cells.

In Vivo Antitumor Efficacy of Mu-HSSKLQ//L12ADT Prodrug

On the basis of the pharmacokinetic data presented above, mice (n = 7) bearing LNCaP xenograft tumors were injected intravenously with 7 mg/kg of prodrug daily for three cycles of 5 days each (on days 1–5, 14–18, and 35–39). Control mice (n = 7) received injections of vehicle only. Tumor volume was determined at the times indicated in Fig. 6, A, over a total of 42 days. Tumor growth in the prodrug-treated group was statistically significantly less than that in the control group over the treatment period ($\Pi^2(3) = 33.04$; $P<.001$) (Fig. 6, A). In addi-

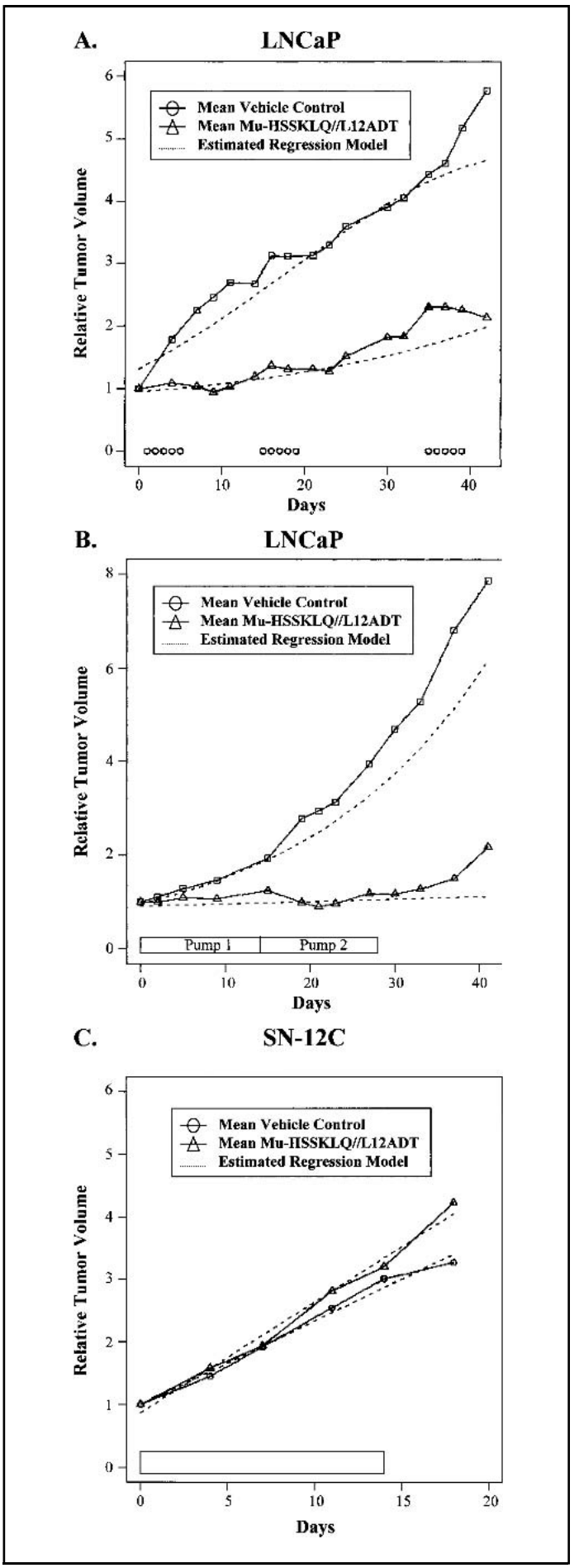

Fig. 6. *In vivo* effect of Mu-HSSKLQ//L12ADT prodrug on xenograft tumors. **A**) Mice bearing prostate-specific antigen (PSA)-producing LNCaP xenografts were treated with three courses of five daily intravenous injections with prodrug (n = 7) or vehicle (n = 7). Relative change in tumor volume was calculated by dividing tumor volumes measured during the course of therapy by the initial tumor volume (i.e., volume at day 0) for each individual mouse. Days of intravenous injection are shown as **open circles.** The tumor volumes from prodrug-treated and vehicle-treated mice were statistically significantly different ($\Pi^2(3) = 33.04$; $P<.001$). **B**) Mice carrying PSA-producing LNCaP xenografts and subjected to continuous subcutaneous infusion of Mu-HSSKLQ//L12ADT (n = 9) or vehicle (n = 9). Mice received 28 days of continuous infusion delivered by two 14-day subcutaneously implanted osmotic minipumps (denoted by **bars**). The tumor volumes from prodrug-treated and vehicle-treated mice were statistically significantly different ($\Pi^2(2) = 116.87$; $P<.001$). **C**) Mice carrying PSA non-producing SN12C human renal cell xenografts were treated with a single 14-day continuous infusion of Mu-HSSKLQ//L12ADT (n = 5) or vehicle (n = 5). There was no evidence of statistically significant differences in tumor volumes between control and treated mice ($\Pi^2(2) = 2.98$; $P = 0.22$). In all panels, **dashed lines** represent the fitted random effects linear regression models, on which the statistical results are based.

tion, transient tumor regression occurred following each cycle of therapy.

To determine whether an enhanced antitumor effect could be achieved with continuous exposure to prodrug, LNCaP xenograft-bearing mice (n = 9) received infusions of 7 mg/kg/day of Mu-HSSKLQ//L12ADT prodrug or vehicle control for 28 days via continuous release from subcutaneously implanted osmotic minipumps. Over the 40-day period of observation, tumor growth in the prodrug-treated group was statistically significantly reduced relative to that in the control group ($\Pi^2(2) = 116.87$; $P<.001$), with nearly complete cessation of tumor growth in the prodrug-treated group (Fig. 6, B). As an additional control, a size-matched group of mice (n = 5) bearing PSA non-producing human SN12C renal cell carcinoma xenografts was treated with the same dose of prodrug or vehicle via osmotic pump. No difference was evident in the growth rate of tumors in the two groups ($\Pi^2(2) = 2.98$; $P = .22$) (Fig. 6, C). These results confirm that proteolytically active PSA must be present within the tumor for prodrug activation and subsequent antitumor effect. It is important to note that, in all of these *in vivo* studies, there was no discernible toxicity, no substantial (i.e., <15%) weight loss, and no deaths in the mice treated with prodrug administered either intravenously or via osmotic minipump over the course of treatment.

To determine whether the PSA-selective peptide was required for targeted delivery of sufficiently cytotoxic levels of L12ADT to achieve an antitumor effect *in vivo,* nude mice (n = 10 per group) bearing TSU bladder cancer xenografts, which do not produce PSA, were injected intraperitoneally with the maximal tolerated dose of L12ADT (i.e., 1 mg [1280 nmol] per kg) or with vehicle daily for 10 consecutive days. On a molar equivalency basis, the L12ADT dose was one-third the dose of prodrug (i.e., 7 mg/kg) used in the antitumor experiments described above. Equimolar doses of L12ADT could not be administered due to acute toxicity to mice. Mice were injected intraperitoneally because their tail veins did not tolerate repeated injections of L12ADT. Animals were divided into two groups of 10 so that average starting tumor volumes in the two groups would be equivalent. Two weeks after the last dose of L12ADT was given (i.e., 24 days from start of dosing), there were no statistically significant differences between the volume of TSU xenograft tumors from L12ADT-treated mice (0.346 cm^3) and that from

vehicle-treated (control) mice (0.315 cm^3) (difference = 0.031 cm^3, 95% CI = –0.02 to 0.08 cm^3). These results suggest that direct systemic administration of L12ADT does not result in an antitumor response, most likely because of insufficient intratumoral accumulation of the drug. These results also indicate that the PSA-targeting peptide is required for delivery of adequate cytotoxic levels of L12ADT within PSA-producing tumors.

DISCUSSION

On the basis of the unique biology of prostate cancer, we initially proposed that PSA could be used to target therapies selectively to metastatic prostate cancer sites within the patient *(23).* Such targeting required the identification of a highly selective peptide substrate for PSA *(28).* The Mu-HSSKLQ peptide substrate was chosen for further development on the basis of both its selective PSA hydrolysis and its stability in the serum of men with high PSA levels *(28).* In this article, we report that a cell-impermeant, latent prodrug consisting of this peptide attached to the thapsigargin analog L12ADT was efficiently hydrolyzed by PSA, was stable in human plasma, and was selectively toxic to PSA-producing prostate cancer cells *in vitro.* Systemic administration of the prodrug to mice bearing PSA-producing LNCaP xenograft tumors led to growth inhibition of these tumors without substantial host toxicity.

Several previous attempts have been made to develop prodrugs containing PSA-cleavable peptides that prevent drugs from entering cells. For example, we initially coupled the Mu-HSSKLQ peptide to a cytotoxic doxorubicin analog, leucinyl-doxorubicin, to produce an inactive prodrug *(32,33).* Using this PSA-activated doxorubicin prodrug, we were the first group, to our knowledge, to validate that such a PSA-cleavable peptide could be used to selectively target prostate cancer cells both *in vitro (32)* and *in vivo (33).* Subsequently this concept was confirmed by Defeo-Jones et al. *(39),* who used a different peptide substrate (gyl-XASZQ//SL, where gyl is N-glutaryl, X is 4-hydroxylprolyl, Z is cyclohexylglycyl, and // denotes the site of PSA cleavage) to construct a doxorubicin prodrug. Wong et al. *(40)* also used this gyl-XASZQ//SL–doxorubicin prodrug to demonstrate that approximately 33% of this prodrug is metabolized to free doxorubicin in the plasma of several species following intravenous administration. In a phase I clinical trial, similar conversion of this gyl-XASZQ//SL–doxorubicin prodrug in the blood of patients to free leucine-doxorubicin (68% conversion) and doxorubicin (19% conversion) was also observed *(41).* These data indicate that the gyl-XASZQ//SL peptide sequence is not optimal for selective targeting. By contrast, our preclinical results demonstrate that less than 1% of the Mu-HSSKLQ//L12ADT prodrug undergoes nonspecific hydrolysis in blood.

Even using a PSA-selective peptide that is more stable in blood, we found that doxorubicin is not an optimal toxic agent to activate apoptosis of low-proliferation-rate prostate cancers (Fig. 2, B). Neither paclitaxel nor doxorubicin is effective at inducing apoptosis of prostate cancer cells under the low-proliferation conditions that mimic the cell kinetic state characteristic of lethal prostate cancers in patients (Fig. 2, B). In contrast, thapsigargin and its analogs are able to induce apoptosis, regardless of the proliferative state of the target cells. Because of this ability to kill both proliferating and non-proliferating cells, thapsigargin represents a particularly attractive agent for the treatment of slowly proliferating prostate cancer cells. However, because thapsigargin and its analogs are non-selective cytotoxins, the prodrug approach is required to efficiently target prostate cancer cells and to minimize systemic toxicity.

In addition to the proliferation-independent cytotoxicity of thapsigargin and its analogs, there are a number of additional advantages to the PSA-activated thapsigargin prodrug approach. After being liberated extracellularly from the prodrug by PSA cleavage, L12ADT rapidly enters cells at the site of activation because of its high degree of lipophilicity, with little release into the general circulation, thus minimizing distant side effects. The PSA-activated thapsigargin prodrug approach also overcomes the problem of heterogeneity in the production of the target protease PSA by individual prostate cancer cells within a given metastatic site. The extracellular fluid of human prostate cancer metastases contains high levels (50–500 μg/mL) of enzymatically active PSA *(29).* Therefore, a substantial cytotoxic bystander effect should occur because cleavage of the thapsigargin prodrug in the extracellular fluid of prostate cancer sites should require production of the active PSA enzyme by only a percentage of the prostate cancer cells within any single lesion. By contrast, other targeted approaches, such as monoclonal antibody or viral vector therapies, require that a large proportion, if not all, of the cells within a metastatic site produce the desired antigenic target or are infected by the viral vector for a substantial cytotoxic effect to occur.

A possible limitation of the use of PSA-cleavable thapsigargin prodrugs is that they could also be activated within the normal prostate. If such activation occurred, however, it should produce minimal problems because removal of the prostate gland via surgical prostatectomy does not affect men's overall health. Similarly, androgen ablation induced by either surgical or medical means activates apoptosis of normal as well as malignant prostate cells without producing toxic effects. Therefore, this potential limitation is unlikely to be clinically significant.

Based on these preclinical studies, this PSA-activated thapsigargin prodrug approach is currently being developed for testing in clinical trials as treatment for metastatic prostate cancer.

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NOTES

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