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Pharmacokinetics, Biodistribution, and Antitumor Efficacy of a Human Glandular Kallikrein 2 (hK2)-Activated Thapsigargin Prodrug

Samuel Janssen,[1] D. Marc Rosen,[1] Rebecca M. Ricklis,[1] Craig A. Dionne,[2] Hans Lilja,[3] Soeren B. Christensen,[4] John T. Isaacs,[1] and Samuel R. Denmeade[1]*

[1]*The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, Baltimore, Maryland*

[2]*GenSpera, Inc., Downingtown, Pennsylvania*

[3]*Memorial Sloan Kettering Cancer Center, New York, New York*

[4]*The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark*

BACKGROUND. Prostate cancer cells secrete unique proteases such as prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) that represent targets for the activation of prodrugs as systemic treatment of metastatic prostate cancer. Previously, a combinatorial peptide library was screened to identify a highly active peptide substrate for hK2. The peptide was coupled to an analog of the potent cytotoxin thapsigargin, L12ADT, to generate an hK2-activated prodrug that was efficiently hydrolyzed by purified hK2, stable to hydrolysis in human and mouse plasma in vitro and selectively toxic to hK2 producing prostate cancer cells in vitro.

METHODS. In the current study, toxicology, pharmacokinetics, prodrug biodistribution, and antitumor efficacy studies were performed to evaluate the hK2-activated prodrug in vivo.

RESULTS. The single intravenous maximally tolerated dose of prodrug was 6 mg/kg (i.e., 3.67 μmole/kg) which produced peak serum concentration of ~36 μM and had a half-life of ~40 min. In addition, over a 24 hr period <0.5% of free L12ADT analog was observed in plasma. The prodrug demonstrated significant antitumor effect in vivo while it was being administered, but prolonged intravenous administration was not possible due to local toxicity to tail veins. Subcutaneous administration of equimolar doses produced lower plasma AUC compared to intravenous dosing but equivalent intratumoral levels of prodrug following multiple doses.

CONCLUSIONS. The hK2-activated prodrug was stable in vivo. The prodrug, however, was rapidly cleared and difficult to administer over prolonged dosing interval. Additional studies are underway to assess antitumor efficacy with prolonged administration of higher subcutaneous doses of prodrug. Second-generation hK2-activated thapsigargin prodrugs with increased half-lives and improved formulations are also under development. *Prostate 66: 358–368, 2006.*

KEY WORDS: **human glandular kallikrein 2 (hK2); prodrug; thapsigargin; SERCA**

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*Correspondence to: Samuel R. Denmeade, MD, The Bunting Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, MD 21231. E-mail: denmesa@jhmi.edu

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INTRODUCTION

Metastatic androgen independent prostate cancer cells have a remarkable low rate of cell proliferation [1,2]. This low proliferative rate could explain the relative resistance of these cells to standard anti-proliferative chemotherapy, while highly proliferative androgen independent prostate cancer cell lines remain exquisitely sensitive to apoptosis induction in vitro [2]. In contrast to agents that activate apoptosis in proliferating cells, our laboratory has shown that thapsigargin (TG), a potent inhibitor of the sarcoplasmic/endoplasmic reticulum ATP-dependent Ca^{2+} (SERCA) pumps [3], has the dose-response ability to elevate intracellular calcium (Ca_i) to sufficient levels to induce apoptosis in all of the rodent and human androgen independent prostate cancer cell lines without requiring the cells to be proliferating [4–7]. The cytotoxicity of TG, however, is not prostate cancer cell type specific [7,8]. Therefore, TG can not be administered systemically without significant side effects. In addition, TG is sparingly water soluble due to its high lipophilicity. Therefore, both to better solubilize TG as well as selectively targets it's cytotoxicity, we have coupled potent TG analogs to peptide carriers to produce inactive prodrugs that are only activated by the prostate tissue specific proteases PSA and hK2 present within extracellular fluid of prostate cancers [7,9,10].

PSA and hK2 are only produced in high levels by normal and malignant prostate cancer cells [11–14]. In addition, metastatic prostate cancer cells continue to secrete enzymatically active PSA and hK2 into the extracellular fluid at high levels [11,12,15]. Once in the extracellular fluid, enzymatically active PSA and hK2 eventually enter the blood where they are inactivated by binding to major serum protease inhibitors [i.e., α1-antichymotrypsin and α2-macroglobulin for PSA [14,16–19] and α1-antichymotrypsin, α2-antiplasmin, antithrombin II, protein C inhibitor, and α2-macroglobulin for hK2 [20,21]. Therefore, PSA- and hK2-activated prodrugs can be administered systemically via the blood without being activated in the circulation due to proteolytic inactivation of PSA and hK2 by abundant serum protease inhibitors.

In previous studies, we synthesized and characterized a series of primary amine containing TG analogs and identified one, 8-*O*-(12-[L-Leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (L12ADT) that is a highly potent inhibitor of the SERCA pump and as cytotoxic as TG [22,23]. Previously, this potent TG analog has been coupled to a PSA-selective peptide to produce a prodrug that is selectively cytotoxic to PSA-producing prostate cancer cells in vitro [7,24]. Significant antitumor effects have also been observed when these PSA-activated prodrugs have been given to animals bearing PSA-secreting prostate cancer xenografts without producing significant host toxicity [24,25].

In an additional study we screened a random, fluorescence quenched combinatorial peptide library and identified a peptide with the sequence acetyl-Gly-Lys-Ala-Phe-Arg-Arg (Ac-GKAFRR) that was a readily hydrolyzed by hK2 with k_{cat}/K_m ratio of $>41{,}000/s^{-1}$ M [10]. This peptide was coupled to L12ADT to generate the hK2-activated thapsigargin prodrug Ac-GKAFRR-L12DT. This prodrug was stable to hydrolysis in human and mouse plasma and efficiently hydrolyzed by hK2 to generate the TG analogs RL12ADT and L12ADT in a ratio of 1:1.8, Figure 1 [10]. The L12ADT has an IC_{50} against prostate cancer cell lines of ~30 nM while the RL12ADT analog is ~5-fold less potent [13]. The prodrug was selectively toxic to hK2 producing prostate cancer cells in vitro with IC_{50}

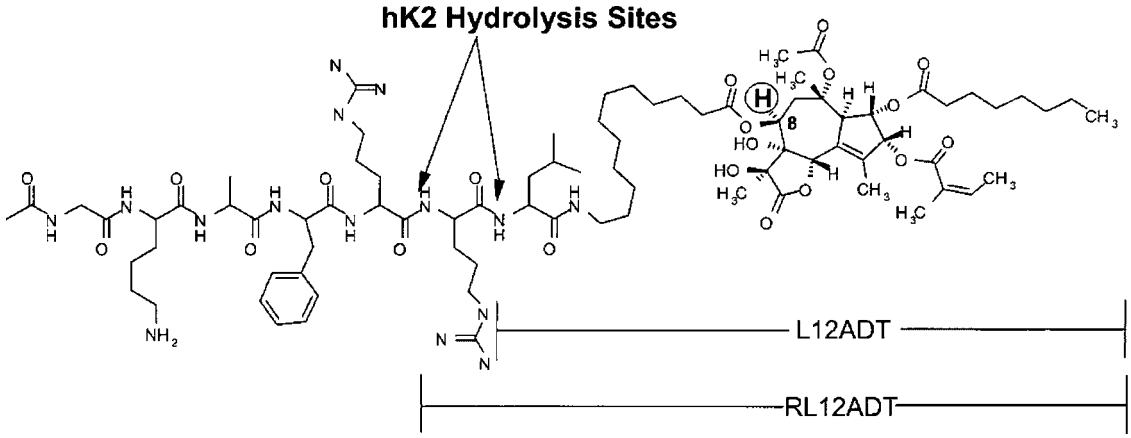

Fig. 1. Chemical structure of hK2 prodrug, Ac-GKAFRRL-12ADT. HK2 cleavage sites are indicated. The ratio of RL-12ADT: L-12ADT generated by hK2 digestion was 1:1.8. Site of ^3H label indicated by circled "H" at 8-position of thapsigargin nucleus.

values of 0.5–1 μM against hK2-producing human prostate cancer cells [10]. In the present study, we have performed additional studies to characterize this hK2-activated prodrug in vivo. Toxicology studies were performed to determine the maximally tolerated dose which was then used for pharmacokinetic studies. Radiolabeled prodrug was synthesized to evaluate biodistribution following single and multiple intravenous doses. Finally, antitumor efficacy studies were performed against hK2-producing LNCaP human prostate cancer xenografts.

MATERIALS AND METHODS

Materials

^{3}H 8-*O*-debutanoylthapsigargin (dBTG) was prepared as previously described [26] and provided through cooperative agreement with NCI-RAID program. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Lines

The LNCaP human prostate cancer line was obtained from ATCC (Rockville, MD); CWR22Rv1 was provided by Dr. Jacobberger (Case Western Reserve University, Cleveland OH). These lines were maintained by serial passage in RPMI 1640 media (Gibco) containing 10% fetal bovine serum (FBS). All standard media included 100 U/ml penicillin G and 100 U/ml streptomycin sulfate (M.A. Bioproducts, Walkerville, MD) and all cells were grown in 5% CO_2/95% air at 37°C.

In Situ Zymography

Zymography was performed according to method of Galis et al. [27]. Briefly, a fluorescence quenched hK2 peptide substrate with the sequence Abz-GKAFRR-LY(NO_2) (where Abz is aminobenzoate) was synthesized as previously described [10]. This peptide was mixed in solution with 1% low melt agarose in 50 mM Tris, 0.1 M NaCl, pH 7.8 to final concentration of 500 μM with and without Complete protease inhibitor cocktail (Roche Labs). Agarose was placed on single glass well microscope slide and allowed to gel overnight at 37°C. The following day, a CWR22R xenograft tumor was harvested and immediately frozen in liquid nitrogen. Frozen section were then obtained, inverted, and applied to agarose gel with slides clipped together and incubated at 37°C in a humidified incubator for 36 hr. This slide ''sandwich'' was evaluated over multiple time points using fluorescence microscope (Zeiss) equipped with DAPI filter set and images obtained.

Synthesis of Prodrugs

Prodrug was synthesized using previously described methods [10]. Semi-preparative HPLC yielded Ac-GKAFRR-L12ADT, typically in 60%–70% yield. The product identity was confirmed by MALDI-TOF analysis. For ^{3}H labeled prodrug, the thapsigargin analog 8-*O*-(12-Aminododecanoyl)-8-*O*-debutanoylthapsigargin (12ADT) was synthesized by coupling 12 aminododecanoic acid to ^{3}H dBTG mixed with unlabeled dBTG in a 1:50 molar ratio as previously described [23] to generate mixture of ^{3}H 12ADT/12ADT. This mixture was then coupled to Ac-GKAFRRL peptide using previously described methods [10]. Prodrug was purified using semi-preparative HPLC coupled to inline radioactive flow detector. Product was confirmed by MALDI-TOF analysis.

Determination of Plasma Levels of Ac-GKAFRR-LI2ADT Prodrug

Calibration standards consisted of Ac-GKAFRR-L12ADT prodrug, RL12ADT or L12ADT spiked into mouse plasma. Plasma samples from treated mice were analyzed by liquid chromatography coupled to a quadripole mass spectrometer (LC/MS/MS) [PE Sciex API 3000]. A multistep gradient elution HPLC method was used to separate the Ac-GKAFRR-L12ADT prodrug from the free RL12ADT and L12ADT with eluent A = 1% acetic acid in deionized water and eluent B = 90% acetonitrile/1% acetic acid/deionized water. Samples were eluted through a Zorbax SB-C18 Rapid Resolution column (2.1 × 30 mm, 3.5 μm) at a flow rate of 0.3 ml/min and gradient of 100% A to 100% B over 12 min. Calibration was done using standards of Ac-GKAFRR-L12ADT added to and then extracted from mouse plasma in a range of 0.001–10 μM. Linear regression analysis was used to generate best-fit lines, from which peak areas of samples were converted to concentration of prodrug. Peak areas of RL12ADT and L12ADT from plasma samples were below limit of detection (i.e., <1 nM) at all time points and, therefore, calibration was not performed. Single-dose pharmacokinetics were assessed by noncompartmental analysis [28]. The area under the curve from time zero to infinity (AUC0-8) was calculated with the linear trapezoidal method [28]. The terminal half-life ($T_{1/2}$) was determined from the terminal slope (ke) on a log-linear plot of concentration versus time.

Red blood cell lysis assays: heparinized blood samples were obtained from discarded samples from clinical chemistry lab. Red blood cells were separated from plasma by centrifugation and then washed with 5 volumes of phenol red free Hanks balanced salt solution (HBSS). Washed cells were then resuspended in either HBSS or 25% (v/v) plasma in HBSS to

concentration of 1.9% red blood cells. Prodrug added from DMSO stock and additional DMSO added to produce final DMSO concentration of 1.9%. After 2 hr incubation at room temperature, red blood cells were pelleted by centrifugation and absorbance of supernatant at 540 nm determined in 96-well spectrophotometer (where) 1% Triton/HBSS used as positive control (i.e., 100% lysis) and data plotted as ratio of absorbance of prodrug versus Triton treated red blood cell supernatants.

In Vivo Toxicity Assays

To determine in vivo toxicity of Ac-GKAFRR-L12ADT, Balb-C mice (Harlan) received a single intravenous injection of an increasing dose of prodrug. Mice were monitored for toxicity hourly for 12 hr and then daily × 1 week. Separate groups of three mice each received increasing doses of Ac-GKAFRR-L12ADT. Dose escalation was stopped at the dose level that resulted in death of all mice after 24 hr (i.e., LD_{100}). All animals receiving doses less than LD_{100} were alive and well up to 1 week after receiving a single dose.

Biodistribution Studies

2×10^6 CWR22R cells in 100 μl of Matrigel (Collaborative Research, Bethesda, MD) were inoculated into the flank of 6-week-old male nude mice (Harlan). Animals were grouped so that the average starting tumor volumes (i.e., ~0.1–0.2 cc) were equivalent. CWR22R tumor bearing nude mice then received intravenous injection of ^3H labeled prodrug. Mice were sacrificed at indicated time points and tumors and organs harvested and weighed. Tissues were dissolved in Tissue Solubilizer BTS-450, a quaternary ammonium hydroxide in toluene (Beckman-Coulter, Inc., Fullerton, CA) at a ratio of 1 ml BTS-450 to each 100 mg of tissue and heated to 50°C overnight in 20 ml glass scintillation vials. Upon cooling, 70 μl of glacial acetic acid was added to eliminate chemiluminescence, followed by 10 ml of Ready Organic scintillation fluid (Beckman-Coulter, Inc.). The samples were counted in a liquid scintillation counter (LS 6000, Beckman-Coulter, Inc.) and concentration of the ^3H in samples was calculated based on calibration curves of spiked control tissues processed as above. Calibration was done using standards in a range of 0.001–100 μM and linear regression analysis used to generate best fit line from which ^3H counts of samples were converted to concentration of prodrug.

In Vivo Efficacy Studies

2×10^6 LNCaP cells in 100 μl of Matrigel (Collaborative Research, Bethesda, MD) were inoculated into the flank of 6-week-old male nude mice (Harlan). Animals were grouped so that the average starting tumor volumes (i.e., ~0.1–0.2 cc) were equivalent. When tumors reached 0.1–0.2 cm^3 size, tumor bearing animals were administered a 6 mg/kg/day dose of Ac-GKAFRR-L12ADT (2% DMSO/H_2O) via daily tail vein injection. Animals were treated once a day for 4 consecutive days. Controls were similarly treated with vehicle only (2% DMSO/H_2O). Tumors were measured with calipers and animals were weighed biweekly while on treatment. At the end of the experiments, animals were sacrificed by CO_2 overdose and tumor weights were obtained. All animal studies were performed according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

RESULTS

hK2 Activity In Vivo

A number of human prostate cancer cell lines are available that produce varying amounts of hK2 (i.e., LNCaP, CWR22R,C4-2B, LAPC-4) [29]. In previous studies Kumar et al. [30] characterized forms of hK2 produced by LNCaP cells in vitro. This group demonstrated that in the absence of synthetic androgen 100% of hK2 in conditioned media exists in the pro-hK2 form. In contrast, in the presence of synthetic androgen, pro-hK2 was found to be fully processed to hK2 [30]. In a related study, Vaisanen et al. [31] demonstrated that in the presence of serum containing media, hK2 and PSA were completely inactive in conditioned media. However, in the serum free media, hK2 was active as evidenced by its ability to cleave the fluorescent substrate PFR-AMC [31].

While these studies have demonstrated that hK2 produced in vitro by human prostate cancer cell lines can exist in an active form under the correct culture conditions, no information is available as to the activity of hK2 produced by xenografts in vivo. Since the goal of these studies is to develop an hK2-activated prodrug that can be administered systemically as treatment for prostate cancer, it was important to establish whether xenografts models produce enzymatically active hK2 in vivo. Since antibodies to discriminate free and total hK2 in the blood were unavailable to us, we developed an in situ zymography (ISZ) method to image hK2 activity in xenografts, Figure 2. In a previous study, we used a combinatorial peptide library to identify a fluorescence quenched substrate that was very efficiently hydrolyzed by purified hK2 [10]. For the ISZ studies we mixed this quenched peptide with the sequence Abz-GKAFRRLY(NO_2) into 1% agarose to form a gel. Frozen sections of a harvested CWR22R xenograft tumor were prepared and applied to the fluorescence quenched peptide containing agarose gel.

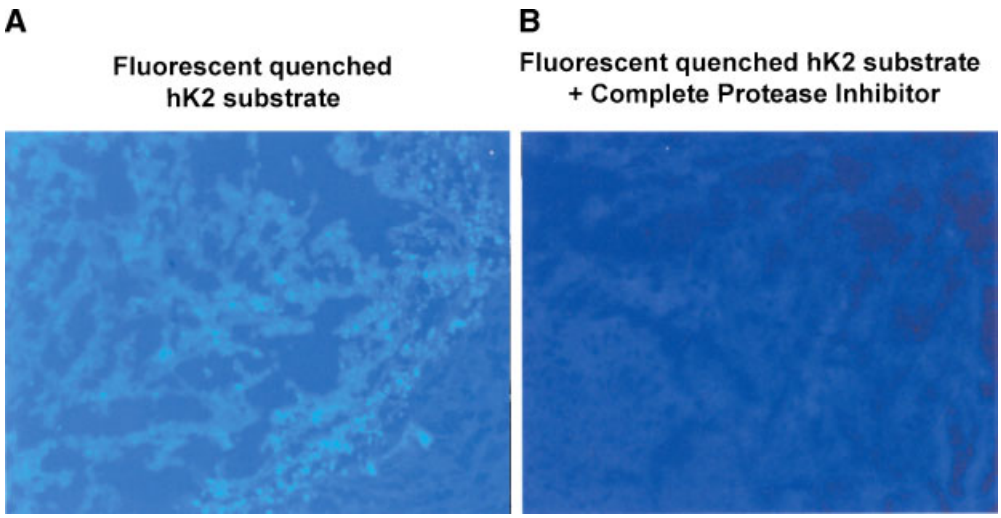

Fig. 2. In situ zymography of hydrolysis of fluorescence quenched Abz-GKAFRRLY(NO_2) peptide. Slides prepared as described in "Materials and Methods." **A**: Fluorescent microscopy image of frozen section of CWR22R xenografts juxtaposed to substrate laden agarose. Fluorescent cords of CWR22R cells can be easily seen at 10× using DAPI filter set. **B**: Fluorescent microscopy image of frozen section of CWR22R xenografts juxtaposed to agarose containing fluorescent substrate and Complete Protease inhibitor cocktail. In this image no significant fluorescence is observed compared to control section containing no fluorescent quenched substrate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Images taken with fluorescent microscope after 36 hr incubation demonstrated increased fluorescence signal surrounding all tumor cells within the xenograft, Figure 2. In contrast, no significant fluorescent signal could be detected in preformed gel containing Complete protease inhibitor cocktail and fluorescence quenched peptide. These results document that hK2 or an hK2-like protease is present in extracellular fluid of these xenografts, which should be able to proteolytically activate the hK2-thapsigargin prodrug containing the same peptide sequence.

Pharmacokinetics and Toxicity Studies

Prior to performing antitumor efficacy experiments with the hK2 prodrug we needed to establish a dose that would be tolerated with minimal toxicity to the animal. To accomplish this, Balb-C mice were treated in groups of three with a single intravenous injection of increasing doses of the Ac-GKAFRR-L12ADT prodrug to establish the dose that killed 100% of mice (i.e., LD_{100}) at 24 hr post dosing. In these studies the LD_{100} was determined to be 18 mg/kg (i.e., 11 μmoles/kg). All mice, however, tolerated a single intravenous dose of 6 mg/kg (i.e., 3.67 μmoles/kg) and this dose was then used for further dosing and pharmacokinetic studies. An additional group of mice ($n = 8$) received four consecutive daily intravenous injections with 6 mg/kg prodrug without any deaths or observable systemic toxicity (i.e., weight loss <15% over baseline) with the exception of significant sclerosis of tail veins.

To determine pharmacokinetic parameters for intravenous administration of the hK2-activated thapsigargin prodrug, Balb-C mice ($n = 3$/timepoint) were treated with a single intravenous dose of 6 mg/kg of the Ac-GKAFRR-L12ADT prodrug. At various time points (5, 10, 30 min and 1, 1.5, 2, 3, 4, 6, 12, 24 hr) mice were sacrificed after blood was obtained by cardiac puncture. After precipitating serum proteins with acetonitrile, supernatants were evaluated by LC-MS to determine concentrations of Ac-GKAFRR-L12ADT, R-L12ADT, and L12ADT at each time points. Areas under the curve were converted to concentrations based on a standard curve that was linear for concentrations ranging from 1 to 10,000 nM. In this study, the C_{max} occurred at 10 min post injection and was 36.8 ± 7.2 μM, Figure 3A. The half-life of the prodrug was 40.7 ± 1.2 min and the area under the curve was 2444.8 ± 39.1 μmol · min/L, Figure 3. Both RL12ADT and L12ADT were below the lower limit of detection (i.e., <1 nM) for all time points, Figure 3A. On the basis of these studies, we concluded that the Ac-GKAFRR-L12ADT prodrug is highly stable to hydrolysis in the serum in vivo.

On the basis of the pharmacokinetic studies, the acute toxicity of the Ac-GKAFRR-L12ADT at higher doses did not appear to be related to conversion of the prodrug to the cytotoxic TG analogs L12ADT and RL12ADT in plasma. Given the amphipathic nature of the prodrug (i.e., positively charged peptide and hydrophobic TG analog), we hypothesized that the toxicity of the prodrug at high doses could be due to formation of micelles at higher concentration. To test

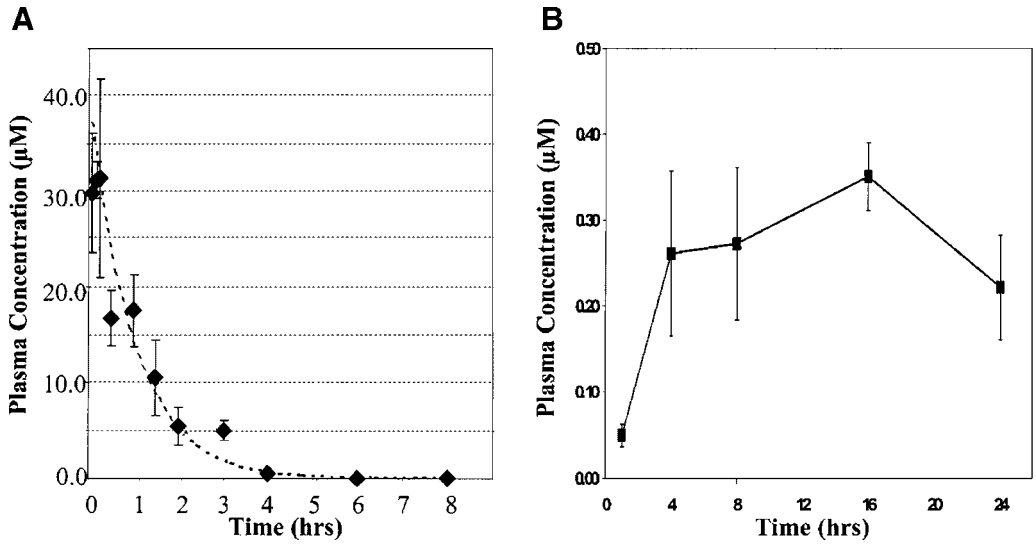

Fig. 3. Pharmacokinetics of Ac-GK AFRRL-I2ADT. **A**: Mice were given single intravenous injection of 6 mg/kg (3.67 μmoles/kg) of prodrug. At indicated times, blood was obtained from anesthetized animals and prodrug extracted from isolated plasma. HPLC analysis was performed and plasma concentrations of prodrug determined by comparison to standard curve. Data represent mean ± standard error of three animals at each time point. Concentrations of cleavage products RL-I2ADT and L-I2ADT were less than I nM at all time points and are not depicted here. **B**: Mice were given a single subcutaneous dose of 6 mg/kg ^{3}H Ac-GK AFRR-LI2ADT. At indicated times, plasma was obtained and processed as described in "Materials and Methods" to determine total counts which were converted to ^{3}H concentration based by comparison to standard curve. Levels represent average ± standard error of plasma from three mice at each time point.

this hypothesis, we evaluated the ability of the prodrug to lyse red blood cells over a range of concentrations, Figure 4. This study demonstrated that the prodrug could effectively lyse RBCs at concentrations = 10 μM when suspended in HBSS but produced no measurable lysis when suspended in 25% human plasma/HBSS up to concentrations of 48 μM. This concentration is 1.3-fold higher than the C_{max} of 36.8 μM observed at the maximally tolerated dose of prodrug of 6 mg/kg. However, this micelle formation could explain the acute toxicity to tail vein following injection of 100 μl of 1 mM dosing solution.

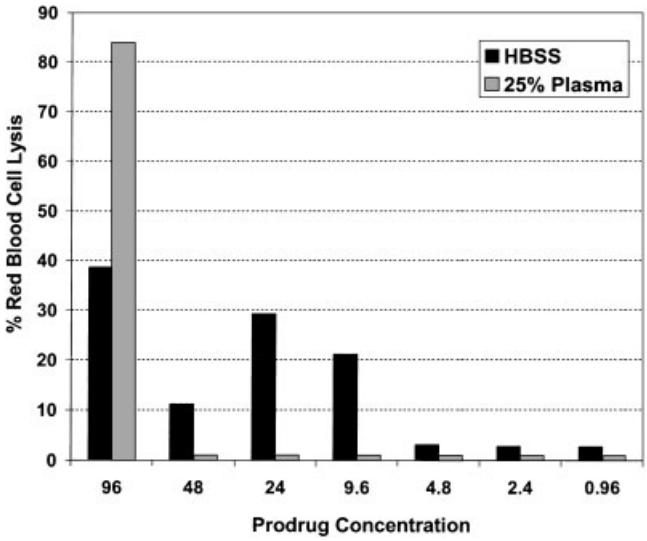

Fig. 4. Lysis of red blood cells by Ac-GK AFRR-LI2ADT. Solution of 1.9% red blood cells suspended in either phenol red free HBSS or 25% (v/v) human plasma/HBSS was prepared as described in "Materials and Methods." Prodrug was then added at indicated concentrations. Ratio of absorbance of prodrug treated cells versus absorbance of 1% Triton treated cells (i.e., 100% lysis of red blood cells) determined and plotted as percent lysis.

To circumvent this local toxicity following intravenous injection, we next determined that the prodrug was unable to form micelles when suspended in 100% propylene glycol. To assess toxicity, mice were given increasing concentrations of ^{3}H prodrug suspended in propylene glycol. A dose of up to 160 mg/kg (i.e., 100 μmoles/kg) (i.e., 27-fold higher than the maximally tolerated intravenous) produced no significant acute toxicity (data not shown). Therefore, to compare pharmacokinetics of the intravenous vs. subcutaneous route of administration, tumor bearing mice (n = 3 per time point) were next given a subcutaneous injection of 6 mg/kg dose of ^{3}H prodrug suspended in 100% propylene glycol to determine if prodrug would be released from the subcutaneous injections site slowly over time producing a ''depot'' effect with lower C_{max}, but sustained release resulting in increased AUC, Figure 3B. In this study, the C_{max} was lower at 350 nM at 16 hr. The AUC however, of 435.1 ± 38.6 μmol · min/L was ~6-fold lower than that observed following single intravenous dosing, Figure 3B. These results suggest that higher subcutaneous doses of Ac-GKAFRR-L12ADT must be given to reach an AUC that

is comparable to single equimolar intravenous dose. However, the toxicity studies suggest that such higher subcutaneous doses could be administered without acute toxicity to animals.

Biodistribution of the hK2-Activated Prodrug

In a previous study, we demonstrated that the Ac-GKAFRR-L12ADT prodrug had an IC_{50} (i.e., concentration that inhibited growth by 50%) of ~0.5–1.0 μM against a panel of hK2-producing human prostate cancer cell lines (i.e., LNCaP, CWR22R, C4-2B) [13]. Given the short half life of the prodrug of ~40 min, in the next set of experiments we wanted to determine if adequate intratumoral levels of the prodrug could be achieved to effect cell killing in vivo.

For these experiments ^{3}H labeled Ac-GKAFRR-L12ADT prodrug was synthesized by coupling ^{3}H dBTG to 12 amino dodecanoic acid to generate the thapsigargin analog 12ADT. This ^{3}H labeled analog was then coupled to the Ac-GKAFRRL peptide to generate Ac-GKAFRRL-(^{3}H)12ADT which was purified by HPLC. Since the ^{3}H label is on the 8-position of the 12ADT thapsigargin analog, any counts measured in tissue must be due to the presence of either the cleaved, active L12ADT analog or the uncleaved, inactive prodrug, Figure 1.

In the first set of experiments, mice bearing CWR22R xenografts were given a single 6 mg/kg dose of Ac-GKAFRR-L12ADT and organs, tumor, and other materials (i.e., stool, urine) obtained at indicated time 1, 4, and 24 hr post inoculation, Figure 5. As observed in the single dose pharmacokinetic study described above, plasma levels of the prodrug fell rapidly over 24 hr. The highest levels of ^{3}H were found in the liver (i.e., 33 μM at 1 hr) which declined rapidly to ~1.1 μM at 24 hr post dosing, Figure 4. The majority of the Ac-GKAFRR-L12ADT appeared to be cleared through biliary excretion as evidenced by high levels of prodrug (i.e., >10 μM) in the stool 4 hr after administration. A significant, although lower, amount of activity was also present in the urine where peak concentration of prodrug was observed 1 hr after dosing. Intratumoral concentrations reached levels comparable to the in vitro IC_{50} of the prodrug with concentrations of 1.25 μM seen at 1 and 4 hr. Intratumoral concentrations (i.e., ~0.9 μM) which are tumorcidal in vitro were still present at 24 hr post-dosing, Figure 5.

Since toxicology studies demonstrated that mice could tolerate repeat daily doses of Ac-GKAFRR-L12ADT, the next experiment was to determine if the prodrug could accumulate in tumor tissue with repeat dosing. For this study, mice bearing subcutaneous LNCaP or CWR22R tumors (n = 3 per group) were given 6 mg/kg dose daily for up to four consecutive doses. Mice were sacrificed at 24 hr post 1, 2, 3, or 4 doses of prodrug to obtain nadir levels of prodrug, Figure 6A. These studies revealed that the prodrug accumulates to varying degrees in most tissue including tumors. LNCaP intratumoral levels of ^{3}H prodrug increased ~3-fold over four doses (i.e., 1.1–3.0 μM) whereas a more modest 1.5-fold increase was observed in CWR22R tumors (i.e., 1.1–1.5 μM), Figure 6A. Two to threefold increases in ^{3}H prodrug levels were also measured in all other tissues examined in the study (i.e., liver, kidney, spleen, skeletal muscle, and plasma).

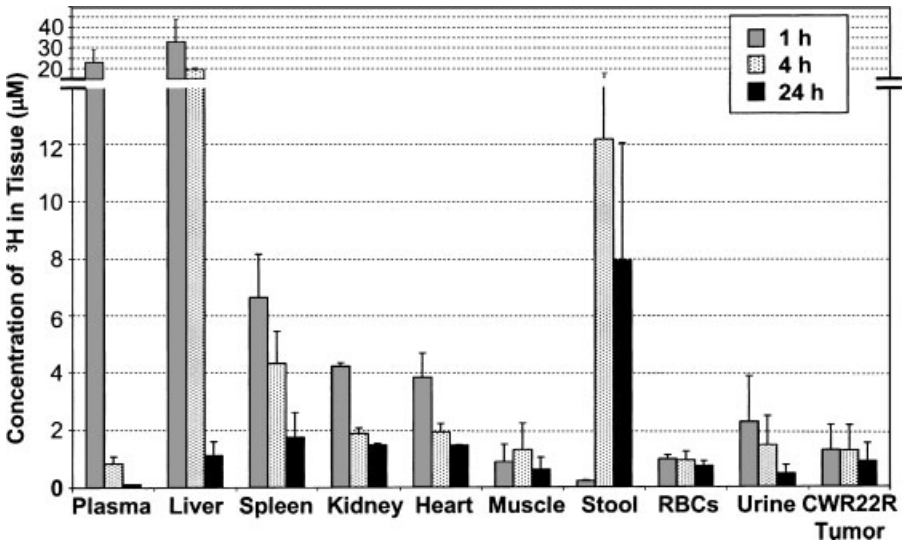

Fig. 5. Biodistribution of Ac-GK AFRR-LI2ADT following single 6 mg/kg intravenous dose. Tissues were harvested at I, 4, and 24 hr post dosing and levels of ^{3}H in tissue homogenates determined and converted to nM based on standard curve. Levels represent average ± standard error of tissue homogenates from three mice at each time point.

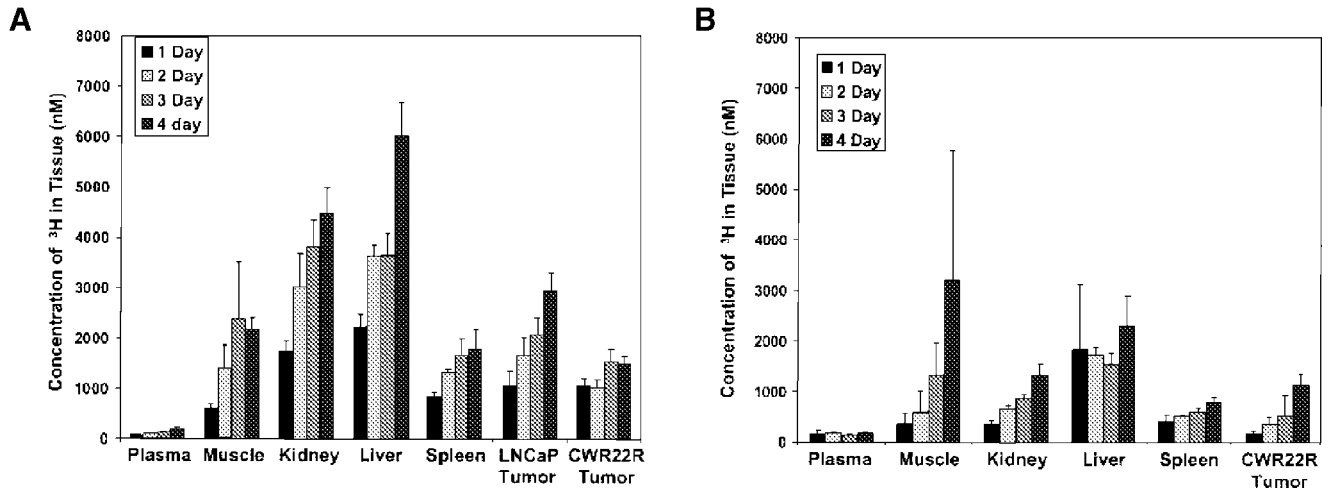

Fig. 6. Biodistribution of Ac-GKAFRR-LI2ADT (**A**) following multiple daily 6 mg/kg intravenous doses. **B**: Following multiple daily 6 mg/kg subcutaneous doses. Tissues were harvested at 24 hr post dosing on days I, 2, 3, 4 and nadir levels of ^{3}H in tissue homogenates determined and converted to nM based on standard curve. Levels represent average ± standard error of non-tumor tissue homogenates from six mice at each time point. For tumors, levels are average ± standard error from three mice at each time point.

Given the local toxicity to tail vein following repeat intravenous dosing, we next evaluated biodistribution of the ^{3}H prodrug when given as a subcutaneous depot formulation in 100% propylene glycol to determine if this form of delivery produced increased area under the curve in a variety of tissues. For this study, mice bearing subcutaneous CWR22R tumors or controls (n = 3 per group) were given daily subcutaneous injections of 6 mg/kg ^{3}H Ac-GKAFRR-L12ADT for up to four consecutive doses. Mice were sacrificed at 24 hr post 1, 2, 3, or 4 doses of prodrug to obtain nadir levels of prodrug, Figure 6B. These studies demonstrated that subcutaneous administration did not result in increased accumulation of prodrug in any tissue compared to daily intravenous bolus administration, Figure 6B. The prodrug, however, did accumulate in CWR22R levels by 6.5-fold (i.e., 0.17–1.1 μM) over 4 days of dosing. In addition, the levels of prodrug observed in CWR22R tumor was comparable after 4 days subcutaneous dosing to levels seen following intravenous dosing over 4 days, Figure 6A,B. High concentrations of ^{3}H prodrug were also measureable in the feces after each daily dose consistent with significant biliary excretion of prodrug following subcutaneous dosing.

In Vivo Antitumor Efficacy of Ac-GKAFRR-LI2ADT Against hK2-Producing LNCaP Xenografts

The biodistribution studies demonstrated that, even though the half-life of the Ac-GKAFRR-L12ADT is short, adequate nadir tumor tissue levels (i.e., 3-fold above in vitro IC_{50}) of prodrug could be achieved after four daily doses of 6 mg/kg of prodrug in LNCaP tumors. In these studies, however, the level of active L12ADT analog in tumor tissue is not known. Therefore, to assess whether adequate growth inhibitory levels of L12ADT are released within the tumor, mice bearing LNCaP tumors were treated with daily intravenous injections of 6 mg/kg prodrug and tumor growth and toxicity was compared to vehicle control (2% DMSO) treated tumor bearing mice, Figure 6A.

After four daily doses of Ac-GKAFRR-L12ADT prodrug, a significant difference in average size of tumors in treated versus control was observed, Figure 7A. Analysis of individual treated mice demonstrated that 9/12 mice had either no growth or tumor regression after four doses and 4/12 mice had ~50% decrease in tumor size relative to starting volume. In contrast, in the vehicle control group only 2/12 animals had no growth at day 4 with one animal demonstrating a decrease from initial size. No toxic deaths were observed in the prodrug treated group. While mice tolerated the therapy as evidenced by no significant loss of body weight (i.e., <5%) Figure 7B, administration of greater than four doses became difficult due to local toxicity with sclerosis of tail veins.

DISCUSSION

The purpose of these studies was to further characterize an hK2-activated thapsigargin prodrug in vivo to determine if adequate levels of prodrug could be administered to achieve an antitumor effect against hK2 producing prostate cancer cells while having minimal systemic toxicity. In this study, we established a maximally tolerated daily intravenous dose in mice of 6 mg/kg. At this dose, the hK2-activated TG prodrug

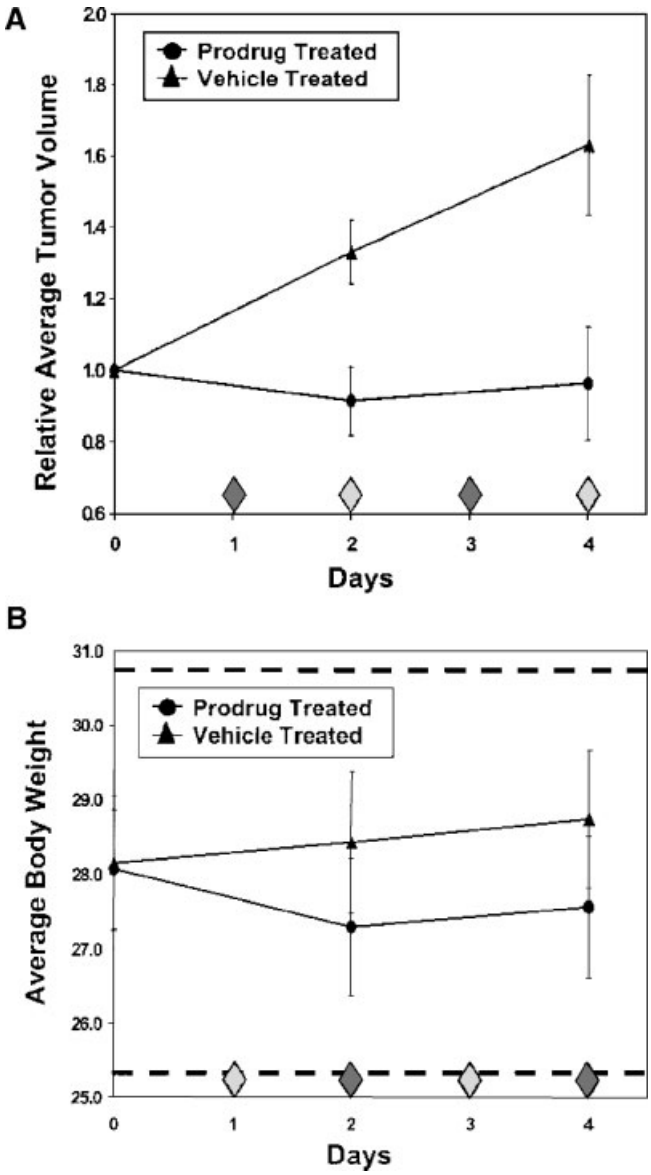

Fig. 7. Antitumor efficacy of Ac-GKAFRR-LI2ADT prodrug against LNCaP xenografts. **A**: Change in relative average tumor volume over time in animals treated with 6 mg/kg intravenous prodrug vs. vehicle control (2% DMSO/H_2O). Days of treatment indicated by gray diamonds. Data represents average ± standard error of tumor volumes from 12 animals each. **B**: Change in weight of animals treated with prodrug or vehicle control over 14 day period. Dotted lines represent average starting weight ± 10%.

produced no significant toxicity to the animal with the exception of tail vein sclerosis with repeat dosing. At this dose, biodistribution studies demonstrated that intratumoral levels of prodrug at or above the in vitro IC_{50} for growth inhibition could be achieved following a single dose with accumulation of prodrug in tumor following multiple daily intravenous injections. In addition, efficacy studies demonstrated an antitumor effect against hK2-producing LNCaP cells, with tumor regression observed during the dosing interval, but regrowth following cessation of dosing. Further intravenous dosing of the drug was limited by local toxicity to tail vein. This toxicity was due to ability of the prodrug to form micelles at concentrations >50 μM in plasma.

This micelle formation could be circumvented in humans through administration of lower concentrations of the prodrug in larger volumes over prolonged infusion time. However, such conditions are not easily recapitulated when treating mice with total blood volume of ~2 ml. Therefore, to determine whether higher doses of prodrug could be administered in the absence of micelle formation the prodrug was suspended in 100% propylene glycol and administered subcutaneously. Using this formulation, the prodrug was much better tolerated and much higher doses (i.e., 27-fold) could be administered without acute toxicity. Administration of an equimolar subcutaneous dose produced much lower plasma levels and lower AUC compared to intravenous dosing. However, equivalent intratumoral levels of prodrug could be achieved following four days of daily dosing. These studies suggest, therefore, that to assess potential antitumor efficacy, prolonged subcutaneous administration of higher concentrations of prodrug may be possible. Currently, sufficient quantities of the Ac-GKAFRR-L12ADT prodrug are being synthesized in order to address this question.

The hK2 prodrug produced a relatively modest antitumor effect when compared to a similar thapsigargin prodrug (i.e., Mu-HSSKLQ-L12ADT) designed to be activated by the proteolytic activity of PSA [10]. On the basis of in vitro studies we would have predicted that the hK2 prodrug would be more active in vivo than the PSA prodrug. The PSA peptide substrate in the previous study had a k_{cat}/K_m ratio of $23.6/s \cdot M$ [7] whereas the hK2 peptide had a k_{cat}/K_m ratio of $41{,}100/s \cdot M$ [10]. The Ac-GKAFRR-L12ADT is hydrolyzed by hK2 much more rapidly than the Mu-HSSKLQ-L12ADT prodrug is activated by PSA. In addition, the PSA peptide sequence was demonstrated to be selectively hydrolyzed by PSA. In contrast, the hK2 peptide sequence can be hydrolyzed also by trypsin and other trypsin-like proteases (i.e., plasmin and urokinase) and the Ac-GKAFRR-L12ADT prodrug was hydrolyzed by plasmin at a sixfold higher rate than by hK2 [10]. Finally, in situ zymography demonstrated that enzymatic activity is present in extracellular fluid that can hydrolyze the hK2 peptide substrate. As this substrate is not specific for hK2, this activity could be due to hK2 and/or other active trypsin-like protease present in the extracellular fluid. In either case, the zymography results suggest that enzymatic activity is present in the tumor microenvironment that could

hydrolyze the prodrug if adequate tissue levels could be obtained.

The biodistribution studies demonstrated that significant amounts of ^{3}H prodrug accumulate within tumors. In these studies we did not determine which ^{3}H species were present (i.e., intact prodrug, free L12ADT analog or degradation products). However, daily nadir levels of up to 3 μM were achieved in the LNCaP tumors after 4 days of dosing. In this case <5% of the prodrug would need to be hydrolyzed to liberate sufficient L12ADT to reach its LD_{50} (i.e., dose that reduces clonal survival by 50%) of 30 nM against LNCaP cells.

CONCLUSIONS

Although hK2 is produced at a lower level than PSA in prostate tissue, the increased production of hK2 in more poorly differentiated cancers coupled with the several orders of magnitude higher enzymatic activity compared to PSA [32] suggest that total hK2 enzymatic activity in the extracellular fluid may be similar or even greater than that of PSA. Therefore, hK2 represents an attractive alternative candidate for prostate targeted prodrug activation therapy. We have developed an hK2-activated thapsigargin prodrug that is efficiently hydrolyzed by hK2 in vitro but has modest antitumor efficacy in vivo against hK2-producing LNCaP xenografts and short serum half-life, even though biodistribution studies suggest adequate intratumoral levels can be obtained following daily intravenous dosing. While the antitumor effect is short-lived in vivo, the hK2 prodrug did produce significant inhibition and regression of majority of treated tumors during dosing interval, suggesting that enhanced antitumor effects could be achieved with chronic administration. However, the dose limiting toxicity of the hK2-activated prodrug was sclerosis of tail veins which limited long term administration. Therefore, additional studies are underway to study alternative dosing methods and to generate improved intravenous drug formulations that could allow for prolonged administration with decreased local venous toxicity. In additional studies, alternative delivery methods such as a prodrug attachment to polyethylene glycol polymers or encapsulation into nanoparticles are also under study.

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