Pegylated Kunitz Domain Inhibitor Suppresses Hepsin-Mediated Invasive Tumor Growth and Metastasis

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Abstract

The transmembrane serine protease hepsin is one of the most highly upregulated genes in prostate cancer. Here, we investigated its tumor-promoting activity by use of a mouse orthotopic prostate cancer model. First, we compared the tumor growth of low hepsin-expressing LnCaP-17 cells with hepsin-overexpressing LnCaP-34 cells. After implantation of cells into the left anterior prostate lobe, LnCaP-34 tumors not only grew faster based on increased serum prostate-specific antigen levels but also metastasized to local lymph nodes and, most remarkably, invaded the contralateral side of the prostate at a rate of 100% compared with only 18% for LnCaP-17 tumors. The increased tumor growth was not due to nonspecific gene expression changes and was not predicted from the unaltered in vitro growth and invasion of LnCaP-34 cells. A likely explanation is that the in vivo effects of hepsin were mediated by specific hepsin substrates present in the tumor stroma. In a second study, mice bearing LnCaP-34 tumors were treated with a PEGylated form of Kunitz domain-1, a potent hepsin active site inhibitor derived from hepatocyte growth factor activator inhibitor-1 (K_{i,app} 0.30 ± 0.02 nmol/L). Treatment of established tumors with PEGylated Kunitz domain-1 decreased contralateral prostate invasion (46% weight reduction) and lymph node metastasis (50% inhibition). Moreover, serum prostate-specific antigen level remained reduced during the entire treatment period, reaching a maximal reduction of 76% after 5 weeks of dosing. The findings show that hepsin promotes invasive prostate tumor growth and metastasis and suggest that active site-directed hepsin inhibition could be effective in prostate cancer therapy. [Cancer Res 2009; 69(21):8395–402]

Introduction

Hepsin is a member of the type II transmembrane serine protease family (1, 2) and was identified as one of the most highly upregulated genes in prostate cancer (3–8). Immunohistochemical staining revealed strong expression in late-stage tumors and metastatic bone lesions (9, 10), suggesting a role of hepsin in tumor progression. Moreover, based on gene expression analysis, hepsin has also been implicated in ovarian cancer (11), renal cell carcinoma (12, 13), and endometrial cancer (14). However, it has proven difficult to clarify the role of hepsin in tumorigenesis. In vitro studies with different types of tumor cells yielded conflicting results on the role of hepsin in cell proliferation (9, 15, 16). Similarly, results from tumor xenograft studies were inconsistent, showing tumor-promoting as well as tumor-inhibiting activities (17, 18).

Perhaps the most compelling in vivo study arguing for a tumorigenic activity of hepsin in prostate cancer came from Klezovitch and colleagues (19, 20). The authors found that overexpression of hepsin in a mouse model of nonmetastatic prostate cancer promoted primary prostate cancer progression and metastasis to several organs. Intriguingly, hepsin overexpression was associated with disruption of basement membranes, suggesting that hepsin activity might be linked to degradation of basement membrane components. This view is consistent with biochemical studies, which identified several hepsin substrates that are functionally linked to basement membrane degradation and cell motility, such as pro–hepatocyte growth factor (pro-HGF), pro–urokinase-type plasminogen activator (pro-uPA), and laminin-332 (21–23). Of note, uPA colocalized with hepsin in metastatic bone lesions of prostate cancer patients (10), suggesting that hepsin may contribute to the formation of active uPA in bone lesions. Based on its prostate cancer–specific expression and potential role in tumor progression, hepsin has become an interesting candidate gene for early prostate cancer detection (24) and therapeutic intervention.

Hepsin enzymatic activity is potently inhibited by Kunitz-type inhibitors including HGF activator (HGFA) inhibitor (HAI)-1 (21) and by various serpins (25). Whereas most inhibitors lack specificity, HAI-1 appears to have a rather narrow specificity profile. Of 17 serine proteases examined thus far, HAI-1 mainly inhibited hepsin, matriptase, HGFA, trypsin, and prostatin (26–29). Structural and functional studies showed that whereas the substrate specificity of HAI-1 is completely contained in the NH_{2}-terminal Kunitz domain-1 (KD1; ref. 29), other HAI-1 domains can negatively regulate the binding affinity (30). Thus, KD1 by itself is a significantly better hepsin inhibitor than HAI-1 extracellular domain (29), raising the possibility to harness its potency and specificity to inhibit hepsin in vivo.

Toward this aim, we generated a PEGylated form of KD1 (KD1-PEG) with improved plasma half-life to neutralize hepsin activity in a mouse orthotopic prostate cancer model with hepsin-overexpressing LnCaP-34 cells. An initial in vivo study indicated that hepsin overexpression strongly promoted invasive growth in the contralateral side of the prostate associated with increased serum prostate-specific antigen (PSA) levels and periaortic lymph node metastasis. All these effects were markedly reduced by a 7-week treatment with the potent hepsin inhibitor KD1-PEG. The implications on the role of hepsin in prostate cancer and as a therapeutic target are discussed.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Reagents and proteins. The synthetic p-nitroanilide substrates S2765 (DiaPharma FXa substrate) and S244 were from DiaPharma. Pro-uPA was from Fitzgerald Industries Int. Pro-HGF, KD1, and hepsin were recombinantly expressed and purified as described (29, 31, 32). Murine HGF full-length cDNA was obtained from the American Type Culture Collection (m8x5g08.r1). The cDNA encoding the protease domain of murine HGF (coding for Val370-Ser653) was fused to the gp67 leader sequence at the NH2 terminus and a (Ala3-His8x)-tag was added to the COOH-terminal Ser653. This construct was inserted into a baculovirus expression vector and expressed in High Five cells and purified as described for human HGF (27).

Expression, purification, refolding, and PEGylation of KD1. KD1 (residues 246-303 of HAI-1B) with an additional NH2-terminal poly-histidine leader sequence was expressed in Escherichia coli, purified, and refolded as described previously (29). Purified KD1 was diazylated against 50 mmol/L K2HPO4 (pH 6.5; PEGylation buffer) overnight at 4°C. KD1 was mixed with the PEGylation reagent (average MW 28,737 Da; SUNBRIGHT MEGC-30TS; NOF) at a molar ratio of 1:1.5 (KD1:PEG) and incubated for 90 min in PEGylation buffer. The reaction was stopped by acidification. Clarified solution was loaded onto a Hitrap-SP column equilibrated with 50 mmol/L sodium acetate (pH 5.5). Proteins were eluted using a six-step gradient 0.15 to 0.7 mol/L NaCl in buffer A and fractions were analyzed by SDS-PAGE. Fractions containing mono-PEGylated KD1 were pooled and stored at 4°C. The concentration of KD1-PEG was determined by the BCA method (Pierce) and calculated based on the protein content of KD1 (MW 8,919 Da).

Cell proliferation assay. LnCaP-17 and LnCaP-34 cells (32) were seeded at 1,000 per well in a 96-well plate and cultured in serum-supplemented RPMI 1640. At 0, 3, 6, and 28 days, 20 μL Alamar Blue (Invitrogen) was added and cells were quantified according to the manufacturer’s instructions. Results were normalized to day 0 and expressed as the mean ± SD of six wells.

Matrigel invasion assay. The assay was done essentially as described by Xuan and colleagues (9). DU-145, LnCaP-17, and LnCaP-34 cells (1.5 × 105 per well) were added into 24-well Matrigel-coated Fluoroblok invasion inserts (BD Biosciences) with serum-supplemented medium in the lower chamber. After 48 h at 37°C, cells that migrated to the lower side of the filter were stained with 10 μmol/L Yo-Pro-1 (Invitrogen) and quantified by measuring fluorescence intensity on a Spectramax M5 microplate reader (Molecular Devices).

Enzymatic assay with synthetic substrate. KD1-PEG and KD1 were incubated with 0.25 nmol/L hepsin in HEPES [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L CaCl2, and 0.01% Triton X-100] for 40 min. S2765 (0.2 mmol/L) was added and increase in absorbance at 405 nm was measured on a kinetic microplate reader (Versamax; Molecular Devices). The obtained rates (μOD/min) were converted into micromolar units using the molar absorptivity of 48,000 for S2765.

Pro-uPA activation assay. KD1-PEG and KD1 were incubated with hepsin (1 nmol/L) for 40 min and pro-uPA (100 nmol/L) was added. At 45s intervals, the reaction was stopped by addition of S2765 (0.5 mmol/L), the absorbance increase at 405 nm was measured on a kinetic microplate reader (Versamax; Molecular Devices). The obtained rates (mOD/min) were converted into micromolar units using the molar absorptivity of 180,000 for S2765.

Pro-HGF activation by cell surface–expressed hepsin. Confluent LnCaP-34 cells in 48-well plates were incubated with KD1 and KD1-PEG in serum-free RPMI 1640/0.1% bovine serum albumin for 15 min at 37°C before addition of 125I-labeled pro-HGF (0.01 mg/mL) prepared as described (31). After 3 h at 37°C, aliquots were removed and analyzed by SDS-PAGE (4-20% gradient gel; Invitrogen) followed by exposure to X-ray films.

Orthotopic prostate cancer model with LnCaP-17 and LnCaP-34 cells. LnCaP-17 and LnCaP-34 cells (32) were washed and resuspended in PBS for prostate injection. Eight- to 10-week-old male SCID/bg mice were anesthetized i.p. with ketamine (80 mg/kg) and xylazine (10 mg/kg), shaved, and cleaned with iodine and 70% ethanol. A lower midline abdominal incision was made and the prostate was exteriorized. LnCaP-34 cells (5 × 106) in 50 μL PBS (LnCaP-34 group; 21 animals) or LnCaP-17 cells (LnCaP-17 group; 20 animals) were implanted into the left anterior lobe of the prostate and the abdominal incision was sutured. Animals received a single dose of buprenorphine following surgery to alleviate pain. Body weights were measured daily and serum samples for PSA measurements were obtained by orbital bleeding and stored at −20°C. After 16 weeks, animals were euthanized with CO2 inhalation and prostate tumors were surgically removed, weighed, and cut along the midline to yield the right (contralateral side) and left (ipsilateral side) halves of the prostate. During the study, 2 animals of the LnCaP-17 group and 3 animals of the LnCaP-34 group died of unknown causes, resulting in 18 animals of each group for final analysis. Portions of the tumor were snap-frozen for RNA analysis. Sections of the right and left tumor side were cut perpendicular to the midline for H&E staining and histologic inspection. For ex vivo bioluminescence imaging, the lung, liver, spleen, rib cage, as well as all enlarged periaortic lymph nodes were removed and incubated with 25 mg/mL n-luciferin (Invitrogen) in PBS. Organs were imaged using a cooled intensified CCD system to visualize luciferase signal indicative of metastatic lesions. Positive bioluminescence imaging signals were confirmed by histology.

Orthotopic prostate cancer model with KD1-PEG inhibitor. The same model was used, except that only LnCaP-34 cells were used for prostate injection. After 6 weeks of tumor growth, the tumor-bearing mice were identified by measuring PSA serum levels and by in vivo bioluminescence imaging. Mice maintained under isoflurane anesthesia were injected i.p. with 200 μL of 25 mg/mL n-luciferin followed by imaging on a CCD system. Animals were divided into the control group (n = 16) and treatment group (n = 12), both groups having similar PSA levels and body weights. KD1-PEG in PBS or PBS (control group) was administered daily (10 mg/kg i.p.) up to week 14. Body weights were monitored and no significant changes were found between the groups. One animal of the control group died during the experiment and was not included in the final analysis. At 14 weeks, animals were euthanized and tumors and enlarged lymph nodes were removed. Ex vivo bioluminescence imaging of the excised lymph nodes was done as described above, and metastatic prostatic tumor cells in the lymph nodes were confirmed by histology. Primary tumors were cut along the midline and the contralateral and ipsilateral side of prostate tumors were weighed and processed for histology.

Quantification of PSA. Serum PSA levels were measured by use of a human PSA ELISA kit (Anogen). PSA concentrations in culture medium were measured after treating LnCaP-17 and LnCaP-34 cells for 24 h with 1 μmol/L KD1 or KD1-PEG in serum-supplemented RPMI 1640. The results were normalized to cell numbers and expressed as the mean ± SD of three experiments.

Histology. Histologic examination of prostate tumors and other tissues was done at the conclusion of in vivo studies. Tissues were fixed in neutral-buffered formalin and processed for paraffin embedding. Morphologic analysis was done on H&E-stained 4-μm-thick sections by a pathologist (H.K.).

Pharmacokinetic measurements of KD1-PEG and KD1. Eight- to 10-week-old male SCID/bg mice were injected i.v. with 0.5 mg KD1 or KD1-PEG protein. After 2, 4, 8, 24, 48, and 96 h, blood samples were taken by cardiac puncture (two mice per time point) and the prepared plasma was stored at −20°C. Plasma samples were incubated with murine HGF (which, unlike hepsin, is active in plasma) in 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, and 0.5 mg/mL bovine serum albumin for 45 min. After addition of S2765 (0.5 mmol/L), the absorbance increase at 405 nm was measured. Inhibitor concentrations in each sample were calculated from standard curves with purified KD1-PEG and KD1. The concentration versus time curve was best described by a two-compartment elimination profile of the form C(t) = A*exp[-ln(2)*(t/T1/2)] + B*exp[-ln(2)*(t/T2/2)], with an iterative 1/2 weighting scheme (WinNonlin version 5.0; Pharsight).

Results

In vitro effects of hepsin overexpression. The LnCaP-34 cells were generated to stably overexpress hepsin resulting in 5-fold
increased hepsin cell surface expression and 3-fold increased hepsin enzymatic activity compared with the LnCaP-17 cells, which only express endogenous hepsin at relatively low levels, comparable with the parental LnCaP cells (32). Hepsin overexpression was not associated with nonspecific changes in expression of genes potentially involved in hepsin-associated biological pathways, such as matriptase (32), prostasin, HAI-1, HGF, Met, uPA, and uPA receptor (Supplementary Table S1). Moreover, proliferation of LnCaP-34

Figure 1. *In vitro* effects of hepsin overexpression. A, cell proliferation of hepsin-overexpressing LnCaP-34 cells and low hepsin-expressing LnCaP-17 cells. Cell numbers at 0, 3, 6, and 8 d in culture (d3-d8) were normalized to day 0. B, Matrigel invasion assay. LnCaP-17 and LnCaP-34 cell invasion was normalized to DU-145 cells. C, PSA concentrations in medium of confluent cell cultures.

Figure 2. Growth of LnCaP-34 and LnCaP-17 tumors in a mouse orthotopic prostate cancer model. A, weight of individual prostate tumors after 16 wk of growth. Average weight was 1.2 ± 0.3 g for LnCaP-17 tumors and 2.1 ± 0.2 g for LnCaP-34 tumors (P < 0.02). B, serum PSA levels (mean ± SE) of mice bearing LnCaP-17 tumors (open squares) or LnCaP-34 tumors (filled circles). *, P < 0.05. C and D, prostate sections were H&E stained. LnCaP-17 tumors: poorly differentiated tumors in ipsilateral prostate (cell injection side) with hemorrhage and necrosis (C, left) but normal contralateral prostate without infiltrating tumor cells (C, right). LnCaP-34 tumors: poorly differentiated tumors are present in both ipsilateral prostate (D, left) and contralateral prostate (D, right). ts, tumor stroma; tc, tumor cells; h, hemorrhage; nc, necrosis; ns, normal stroma; gl, normal prostate glands. Bar, 200 μm.
cells was indistinguishable from that of LnCaP-17 cells (Fig. 1A). This result contrasts with an earlier study by Srikantan and colleagues (16) showing that hepsin overexpression resulted in reduced LnCaP cell proliferation. Therefore, we tested additional LnCaP clones with hepsin expression levels that ranged between those of LnCaP-34 and LnCaP-17 cells. However, none of these clones showed altered cell proliferation (data not shown). It is conceivable that hepsin expression levels of LnCaP cells used by Srikantan and colleagues (16) were higher than in our LnCaP-34 cells; in the absence of quantitative hepsin overexpression/activity data on their cell line, we are unable to assess potential reasons for the different results.

Furthermore, compared with the prostate cancer cell line DU-145, Matrigel invasion of LnCaP-17 cells was low (12%) and not statistically increased in hepsin-overexpressing LnCaP-34 cells (14%; P = 0.7; Fig. 1B). Also, PSA production by LnCaP-34 cells remained unaltered from LnCaP-17 cells (Fig. 1C). This suggested that hepsin overexpression did not affect cellular function in vitro.

Effects of hepsin overexpression on tumor growth and metastasis in an orthotopic model of prostate cancer. LnCaP cells were injected into the left anterior lobe of the mouse prostate and tumors as well as metastatic lesions were analyzed after 16 weeks. The LnCaP-34 group had a higher tumor occurrence compared with the LnCaP-17 group (78% versus 61%) and significantly increased tumor weights (1.8-fold; Fig. 2A). After week 9, serum PSA levels increased at a much higher rate in the LnCaP-34 group reaching 2.4-fold higher levels than the LnCaP-17 group at week 16 (Fig. 2B).

Figure 3. Inhibitory potency of KD1-PEG. A, molecular model of the hepsin/KD1 complex based on PDB files 1YC0 and 1Z8G, showing three surface-exposed lysine residues (K276, K255, and K287) available for PEGylation. Their location is outside the KD1-hepsin binding interface as viewed along the substrate binding cleft (catalytic triad residues H57, D102, and S195 are indicated). B, inhibitory activity of KD1-PEG (circles) compared with KD1 (squares) in a hepsin enzymatic assay with S2765 substrate. K_%app values were 0.3 ± 0.02 and 0.4 ± 0.02 nmol/L, respectively. C, inhibition of pro-uPA activation by hepsin. Linear rates of uPA formation at increasing concentrations of KD1-PEG (IC50 0.8 ± 0.11 nmol/L).

Figure 4. In vitro characterization and pharmacokinetics of KD1-PEG. A and B, inhibition of pro-HGF processing by LnCaP-34 cells. Cell cultures were incubated with KD1-PEG (A) or KD1 (B) for 15 min, 125I-labeled pro-HGF was added for 3 h at 37°C, and samples were analyzed by SDS-PAGE under reducing conditions. Hepctrl, hepsin control without inhibitor; control, 125I-labeled pro-HGF control (without hepsin or inhibitor). KD1-PEG and KD1 concentrations ranged from 1 μmol/L to 1.4 nmol/L in 3-fold dilution steps. C, PSA levels in medium of LnCaP-34 cells after incubation with 1 μmol/L KD1-PEG or KD1 for 24 h. D, pharmacokinetics. Mice were injected i.v. with 0.5 mg KD1-PEG or KD1 and plasma samples were collected up to 96 h (2 animals for each time point). Inhibitor concentrations in plasma were determined with an enzymatic assay (n = 2).
The results of the study are summarized in Supplementary Table S2. There was a good correlation between tumor weight and PSA levels (Supplementary Fig. S1). Histologic examination of tumor lesions showed poorly differentiated adenocarcinomas with areas of hemorrhage and necrosis (Fig. 2C and D). The majority of LnCaP-17 tumors were confined to the ipsilateral (left) side of the prostate, where tumor cells were injected at the start of the study. Only 18% of tumors showed invasion into the contralateral (right) side of the prostate; the majority of animals had a normal-appearing contralateral side without any detectable tumor cells (Fig. 2C). In contrast, all of the LnCaP-34 tumors (14 of 14) showed invasion and growth of tumor in the contralateral side of the prostate (Fig. 2D). We performed immunohistochemical staining for E-cadherin and vimentin on contralateral prostate tumors to determine if epithelial-to-mesenchymal transition played a role in the increased invasiveness of hepsin-expressing LnCaP cells. Contralateral tumors showed the same phenotype as ipsilateral tumors and showed membranous staining for E-cadherin and lack of staining for vimentin (Supplementary Fig. S2), indicating the absence of epithelial-to-mesenchymal transition. Furthermore, no differences in tumor morphology, particularly at the tumor-stroma interface, were noted between ipsilateral and contralateral tumors. Although the staining was done on tumors established in the contralateral prostatic lobe, we consider it unlikely that tumors will exhibit epithelial-to-mesenchymal transition as they transverse the midline but then revert back to the original phenotype once a discrete tumor mass has formed.

Both LnCaP-17 or LnCaP-34 cells were engineered to express luciferase at comparable levels (32), allowing us to apply ex vivo bioluminescence imaging for detection of metastatic lesions in local lymph nodes, lung, liver, spleen, and rib cage. Metastatic lesions were only found in periaortic lymph nodes and occurred in 29% of the tumor-bearing mice in the LnCaP-34 group (Supplementary Fig. S3) but were absent in the LnCaP-17 group (Supplementary Table S2). These results suggested that hepsin overexpression enhanced LnCaP-34 cells with increased invasive capacity.

Hepsin RNA levels in LnCaP-34 tumors remained high compared with LnCaP-17 tumors (Supplementary Table S3), comparable with the results from cell culture experiments. Expression of other genes, including the related proteases matriptase and prostasin, as well as HAI-1, HGF, Met, uPA, and uPA receptor, were similar in both tumor groups. Comparable results were obtained with the corresponding mouse genes (Supplementary Table S4). Due to the disappearance of mouse prostate epithelium in the LnCaP tumors, the mouse epithelial-specific genes (e.g., matriptase, prostasin, and HAI-1) were absent in the LnCaP groups. However, mouse stromal genes such as uPA and HGF and their cognate receptors uPA receptor and Met were markedly expressed in both groups (Supplementary Table S4), consistent with the presence of mouse stroma in LnCaP tumors (Fig. 2C and D). In agreement with increased vascularization in areas of tumor growth, expression of the endothelial cell marker PECAM was increased in both tumor groups (Supplementary Table S4). There was no difference between tumor groups in the expression of the proliferation marker human and mouse cyclin D1 (Supplementary Tables S3 and S4).

**Generation and characterization of PEGylated KD1 inhibitor (KD1-PEG).** The HAI-1–derived hepsin inhibitor KD1...
has a short plasma half-life. Therefore, KD1 was PEGylated with 29 kDa PEG chains by use of lysine coupling chemistry. A structural model of the KD1/hepsin complex indicated that three surface-exposed lysine residues of KD1 were available for PEGylation. They were located outside the binding interface, suggesting that attached PEG chains may not substantially interfere with hepsin inhibition by KD1 (Fig. 3A). Purified mono-PEGylated KD1 (KD1-PEG) inhibited hepsin with a $K_{i}^{app}$ of 0.3 ± 0.02 nmol/L, similar to KD1 with a $K_{i}^{app}$ of 0.4 ± 0.02 nmol/L (Fig. 3B). Furthermore, KD1-PEG inhibited the rates of uPA formation in a concentration-dependent manner (Fig. 3C) with an IC$_{50}$ value of 0.8 ± 0.11 nmol/L, similar to KD1 (IC$_{50}$ 0.6 ± 0.03 nmol/L; data not shown). Both KD1-PEG and KD1 inhibited pro-HGF activation by LnCaP-34 cells with similar potencies (Fig. 4A and B). At the fully inhibitory concentration of 1 μmol/L, neither KD1-PEG nor KD1 affected the production of PSA by LnCaP-34 cells (Fig. 4C). Most importantly, KD1-PEG displayed dramatically improved pharmacokinetic properties. In contrast to the rapidly clearing KD1 (below detection at 1 h), KD1-PEG plasma levels could be measured up to 96 h (Fig. 4D) with $T_{1/2a}$ of 3 h and $T_{1/2b}$ of 54 h, comparable with a recently published multi-PEGylated Kunitz domain inhibitor of plasmin (35). As drug levels of KD1-PEG decreased ~50-fold in the first 24 h, a daily dosing regimen of 10 mg/kg was selected for efficacy experiments.

**Effects of KD1-PEG on invasive tumor growth and metastasis.**

LnCaP-34 cells were implanted into the left anterior prostate lobe and the tumors were allowed to grow for 7 weeks before treatment. Histologic examination showed that all tumors were still confined to the ipsilateral side of the prostate (tumor weight 0.26 ± 0.02 g n = 9) without invasion of the contralateral prostate. KD1-PEG was administered daily from weeks 7 to 14. The trough serum levels of KD1-PEG ranged from 122 ± 33 to 180 ± 57 nmol/L (Supplementary Fig. S4), although some samples were below the assay detection limit (2 of 6 at week 8 and 1 of 6 at weeks 10 and 12). This indicated that the majority of treated animals were exposed to KD1-PEG levels expected to substantially inhibit hepsin activity.

At the end of the study, the contralateral prostate tumors of the control group were almost the size of the ipsilateral prostate tumor (Fig. 5A), averaging 83% of the ipsilateral tumor weight (Fig. 5B). KD1-PEG reduced the weights of contralateral prostate tumors by 46% ($P < 0.05$; Fig. 5A and C) and this effect was relatively independent on the ipsilateral prostate size (Fig. 5D). In one animal, the contralateral prostate was actually completely tumor-free (Fig. 5D, asterisk, and Fig. 5A, bottom). KD1-PEG had no effect on the left prostate tumor weight (0.37 ± 0.05 g for controls versus 0.38 ± 0.05 g for KD1-PEG), which may not be surprising, because its average weight only increased ~1.5-fold compared with week 6.

KD1-PEG also strongly suppressed serum PSA levels during the entire experiment (Fig. 6A). One week of dosing already resulted in reduced PSA levels, and at weeks 12 and 14, PSA level reduction reached 76% ($P < 0.05$) and 63% ($P < 0.05$), respectively. In addition, lymph node metastatic lesions occurred in 10 of 15 control animals but only in 4 of 12 KD1-PEG–treated animals, a reduction of 50% (Fig. 6B).

**Discussion**

Despite the strong association of hepsin with prostate cancer based on expression analysis of large numbers of patient samples, a functional role of hepsin in promoting prostate cancer has been difficult to demonstrate. A possible reason is that commonly used mouse xenograft tumor models lack the appropriate prostate-specific microenvironment. This could be of considerable importance, because the epithelial surface-expressed hepsin is ideally located to communicate with the stromal environment, for example, by processing stroma-specific substrates by its surface-exposed protease domain. Based on these considerations, we established an orthotopic prostate cancer model, which showed accelerated growth of the hepsin-overexpressing LnCaP-34 tumors resulting in almost a doubling of tumor weights compared with the low hepsin-expressing LnCaP-17 tumors. This can be attributed to the ability of the LnCaP-34 tumors to invade the contralateral side of the prostate leading to contralateral tumor weights that approached those of the ipsilateral side.

The increased tumor growth was not predicted from *in vitro* assays showing that hepsin overexpression neither increased the invasive capacity nor cell growth. This observation rather points to the potential involvement of tumor stromal components in the hepsin pathway. Specifically, the hepsin-dependent contralateral invasion and growth likely involved degradation of extracellular matrix and basement membranes. In this regard, gene expression analysis showed that genes implicated in degradative and invasive pathways, such as murine uPA and HGF, were expressed in the tumor stroma and could potentially serve as substrates for human hepsin on tumor cells (21, 22, 32). There is similarity to the expression of HGF and uPA in human prostate cancer in that both proteins are expressed in the tumor stroma and have been implicated in tumor progression (36–38). In addition, hepsin was shown to degrade laminin-332, a key component of basement membranes (23). Therefore, it is possible that hepsin-stromal substrate interactions contributed to the locally invasive growth of LnCaP-34 tumors and, perhaps, also to the formation of metastases in periaortic lymph nodes.

Our results support a role of hepsin in tumor progression, in good agreement with conclusions drawn from a genetic mouse model study with prostate-specific overexpression of mouse hepsin (19). However, our orthotopic model showed that hepsin overexpression also resulted in invasive tumor growth leading to increased final tumor weight and elevated PSA levels, whereas tumor growth was not increased in the study by Klezovich and...
colleagues (19). It is possible that, in this latter study, hepsin-dependent effects on primary tumor growth may have been masked by the fast SV40 T antigen–driven tumor growth, whereas our orthotopic model may be more amenable for detecting such effects due to the relatively slow growth of LncAP tumors and the availability of a distinct prostate compartment (the contralateral side of the prostate) for tumor expansion.

Targeting the protease active site is the most direct and common strategy to neutralize protease activity. Here, we used the relatively specific hepsin inhibitor KD1 to examine whether neutralizing hepsin enzymatic activity could suppress tumor progression. Because of the inherently short plasma half-life of Kunitz domain inhibitors, as exemplified by aprotinin (Trasylo), we produced the mono-PEGylated KD1-PEG having greatly improved pharmacokinetic properties. As predicted from a structural model, the attachment of a PEG moiety to KD1 did not reduce its inhibitory potency as determined by various enzymatic assays. The KD1-PEG inhibition study was designed to specifically investigate the effects of neutralizing enzymatic activity during the progressive stages of tumor growth. Based on serum PSA levels, the onset of hepsin inhibition was rapid and persisted throughout the entire treatment period, suggesting strong effects on viable tumor mass. Concordantly, the contralateral prostate tumor weights were significantly reduced, suggesting that KD1-PEG effectively suppressed the hepsin-specific pathways that promoted invasion and growth in the contralateral prostate. However, KD1-PEG did not significantly reduce ipsilateral prostate tumor and overall tumor weights, which may be explained by the fact that, before KD1-PEG treatment, they had already reached 70% and 43%, respectively, of their final weights. This would also imply that hepsin inhibition was primarily effective in suppressing invasion and subsequent growth rather than reducing preexisting tumors. Consistent with this interpretation, KD1-PEG also interfered with the ability of tumors to form metastatic lesions in periaortic lymph nodes.

A caveat with using KD1-PEG as hepsin inhibitor in our tumor model is that KD1 is not completely hepsin-specific. Therefore, we cannot formally rule out the possibility that it also inhibited protease substrates, which underwent hepsin-dependent activation during tumor progression. Such putative protease substrates may be localized to the tumor stroma and/or on the tumor cell surface. It is, however, important to note that KD1-PEG had strong effects on contralateral growth and metastasis, two endpoints that are entirely dependent on high hepsin expression in the tumor. Therefore, when considering potential KD1-PEG “off-target” effects contributing to its inhibitory activity, it is clearly within the context of a hepsin-driven pathway. Only the use of a highly specific inhibitor could provide absolute clarity. In this respect, protein engineering strategies have been developed for increasing Kunitz domain specificities (39) and should be applicable to KD1. Alternative approaches are the use of highly specific anti-hepsin antibodies (9).

The suppression of hepsin-driven tumor progression by KD1-PEG indicates that targeting the hepsin active site could be a promising strategy for prostate cancer therapy. As with any therapeutic intervention, these approaches will carry the risk of adverse effects. Until recently, hepsin deficiency, and thus therapeutic neutralization of hepsin activity, was perceived as safe, because hepsin-deficient mice displayed an apparently normal phenotype (40, 41). However, a recent study by Guiponi and colleagues (42) showed that these mice were severely hearing-impaired. Although this was likely the result of an irreversible developmental defect without any bearing on the adult organism, it nevertheless adds a cautionary note to therapeutic anti-hepsin strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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