A Novel Antitumor Prodrug Platform Designed to Be Cleaved by the Endoprotease Legumain

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Chemotherapeutic treatment of neoplastic diseases is often restricted by adverse systemic toxicity, which limits the dose of drug that can be administered, or by the appearance of drug resistance. Therefore, novel targeted therapeutic approaches are being developed to improve current conventional therapy in order to increase specificity and biocompatibility, and decrease toxicity. Legumain represents a recently identified lysosomal protease that has been reported to be overexpressed in the majority of human solid tumors, to promote cell migration and is associated with enhanced tissue invasion and metastases. Therefore, it serves as a promising candidate for prodrug therapy. We synthesized a novel legumain-cleavable prodrug, carbobenzyloxy-alanine-alanine-asparagine-ethylenediamine-etoposide, which releases the chemotherapeutic agent, etoposide, as the active drug. The prodrug was characterized and analyzed by ^{1}H NMR and HPLC. 293 Human embryonic kidney (293 HEK) cells were stably transfected with human legumain, to achieve overexpression in vitro (293 HEK-Leg). 293 HEK-Leg cells expressed both active and inactive legumain and secreted it to the medium. Legumain expression was found to be elevated because of serum starvation in both 293 HEK cells and PC3 human prostate carcinoma cells. The commercial substrate of legumain, carbobenzyloxy-alanine-alanine-asparagine-amino-4-methyl coumarin (CBZ-Ala-Ala-Asn-AMC) and the synthesized prodrug were both cleaved by recombinant human legumain (rhlegumain) and legumain expressed in the 293 HEK-Leg cell lysate. Upon cleavage by rhlegumain, the prodrug showed an inhibitory effect on the proliferation of 293 HEK and 293 HEK-Leg cells. This study suggests a novel platform for prodrug therapy activated by legumain as a promising approach for cancer therapy.

INTRODUCTION

Current conventional chemotherapy, though widely used in cancer treatments, is still far from reaching its optimal potential. Clinically used chemotherapeutic drugs exhibit a short half-life in the bloodstream and a high overall clearance rate. Most chemotherapeutic drugs are considered to be low-molecular-weight compounds, and as such, they diffuse rapidly and are distributed evenly within the body, into tumor tissues as well as healthy ones (*1*). Thus, small amounts of the drug reach the target cancerous site, whereas healthy tissues suffer from many side effects that limit the maximum tolerated dose (MTD[1]) that can be administered (*1*).

The development of cancer prodrug therapies is implemented to solve the limitations of conventional chemotherapy. Prodrugs have been designed for optimal structure, size, and surface characteristics to increase their circulation time in the bloodstream. They are able to carry their loaded active agents to cancer cells by selectively using the unique pathophysiology of tumors, such as the acidic, neoplastic supporting microenvironment. As such, the microenvironment surrounding the tumor has been found to overexpress specific proteases that are active in these acidic conditions (*2*−*4*). It can be utilized for prodrug-targeting by linking a spacer cleavable by hydrolysis at low pH or by lysosomal or tumor-associated overexpressed enzymes resulting in the release of the active drug.

Legumain is a member of the C13 family of peptidases that specifically cleaves asparaginyl bonds. It was first discovered in plants (*5*−*7*) and later in parasites (*8, 9*) and mammals (*10, 11*). All legumains are synthesized as a 50−60 kDa pro-enzyme that undergoes activation upon autocleavage at acidic conditions (pH 3.0−6.0). Mature, active mammalian legumain varies in size from 30 kDa to 40 kDa, due to different degrees of glycosylation. Chen et al. detected 3 bands of human legumain in an immunoblot from a cell lysate consisting of a 56 kDa inactive pro-enzyme, a 46 kDa active form, and a 36 kDa mature active

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[1] Abbreviations: 293 HEK, 293 human embryonic kidney; 293 HEK-Leg, 293 HEK cells stably transfected to overexpress legumain; ACN, acetonitrile; AMC, amino-4-methyl coumarin; AUC, area under the curve; BCA, bicinchoninic acid; CBZ, carbobenzyloxy; CT26, murine colon carcinoma cells; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethyl formamide; DNA, deoxyribonucleic acid; ECGS, endothelial cell growth supplement; EDTA, ethylene diamine tetraacetic acid; EtOAc, ethylacetate; FBS, fetal bovine serum; FDA, food and drug administration; G418, neomycin; HUVEC, human umbilical vein endothelial cells; HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase; IC_{50}, inhibitory concentration of 50% of the cells; IgG, immunoglobulin G; kDa, kilo Dalton; MeOH, methanol; MES, 2-(*N*-morpholino)ethanesulfonic acid sodium salt; MTD, maximum tolerated dose; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PNP, *p*-nitrophenyl; rhlegumain, recombinant human legumain; RP, reverse phase; t_R, retention time; s.c., subcutaneously; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; TRAMP C2, transgenic adenocarcinoma of the mouse prostate C2; XTT assay, cell proliferation kit based on the tetrazolium salt XTT.

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enzyme. Pro-legumain is autocleaved at asparaginyl residues as well as aspartic ones, upon activation at pH 4.5 to produce the 46 kDa active form. Further autocleavage at acidic conditions results in the mature form of the 36 kDa, active human legumain. Once the enzyme is in its active form, raising the pH level up to 7.0 results in enzyme denaturation and therefore inactivation (*12*). The precise physiological role of mammalian legumain in human biology is still poorly understood. It has been found to be involved in the processing of the microbial tetanus toxin (*13*) and to play a role in osteoclast inhibition (*14*). Furthermore, it has been suggested that legumain is responsible for the processing of cathepsins B, H, and L (*15*) and activating progelatinase A to produce mature active gelatinase A (MMP-2) in cultured cells (*16*). A recently published study has also found legumain to regulate the degradation of fibronectin in renal proximal tubular cells. This implicates legumain in the control of extracellular matrix remodeling and turnover, a well-known process in various pathological conditions such as tumor growth, development of metastases and progression of atherosclerosis (*17*). In recent years, legumain has been reported to be overexpressed in tumors (*18*). High levels of legumain were found in tumor cells, as well as in tumor-associated endothelial cells, tumor-associated macrophages, and other stromal cells. Though never explored or examined empirically, legumain has been indicated to be up-regulated in vitro as a result of stress such as serum starvation or in vivo growth (*19*). Legumain is almost undetectable in vitro, suggesting that its expression is highly dependent on the unique microenvironment of the tumor (*19*). Therefore, we generated a cell line stably overexpressing legumain, in order to characterize its activity in vitro. Legumain is not only highly expressed in the lysosomal cell fraction (*10*), but it is also associated with the cell surface and colocalized with integrin β1. In addition, in vitro assays showed that tumor cells overexpressing legumain exhibit increased migration and invasion through the extracellular matrix. Furthermore, legumain was suggested to play a role in promoting tumor progression and correlated with a more aggressive behavior and a poorer prognosis (*20*). Legumain's unique specificity to cleavage of asparaginyl bonds and its connection to cancer progression led to the interesting notion regarding legumain as a target for various prodrug designs in targeted chemotherapeutics (*19, 21, 22*).

In this study, we designed and synthesized a legumain-activated prodrug of etoposide. Etoposide, 4′-demethyl-epipodophyllotoxin-9-[4,6-*O*-(*R*)-ethylidene-β-D-glucopyranoside], is a semisynthetic derivative of podophillotoxin, classified as a cell cycle-specific, plant alkaloid antineoplastic chemotherapeutic drug. It was the first topoisomerase II inhibitor to be discovered (*23*). Etoposide does not block the activity of this essential enzyme, but rather increases the steady-state concentration of the covalent DNA cleavage complexes, converting them into physiological toxins. High level of DNA breaks arise in the genome, becoming targets for recombination, sister chromatids exchange, insertions, deletions, chromosomal aberrations, and translocations. These alterations trigger a series of events, resulting in cell death and apoptosis, thus killing the tumor cells (*24*). Etoposide is one of the most highly prescribed anticancer drugs in the world, even though it possesses the same characteristics as most chemotherapeutic drugs do. Being a hydrophobic low-molecular-weight compound (588 g/mol), etoposide diffuses through the bloodstream to healthy tissues and causes many side effects. The primary dose-limiting toxicity of etoposide in clinical use is severe myelosuppression (*25*). Etoposide undergoes hydrolysis and epimerization to generate metabolites that are 100-fold and 500-fold less cytotoxic, or not active at all (*26*). Its low water-solubility forces it to either be solubilized in toxic solvents, such as tween-80 or ethanol, or to be diluted to low concentrations and administered in high volumes of an isotonic solution, causing discomfort either way (*27*). Accumulation of DNA breaks, caused by etoposide, generate potential mutations and chromosomal translocations that may also lead to myeloid/lymphoid or mixed lineage leukemia (*28*). Furthermore, it undergoes nonspecific hydrolysis, and it induces drug resistance in repeated treatment. New drug delivery systems are currently being developed in the hope of evading etoposide's limitations and toxicities while preserving and even elevating its antitumor activity, thus increasing its therapeutic index.

A well-established etoposide−prodrug system will prevail etoposide limitations and improve its efficacy and specificity in various cancer treatments. Here, we show a prodrug of etoposide linked to a tripeptide cleavable by legumain. We hypothesize that this prodrug will be activated by the overexpressed legumain in tumor epithelial and endothelial cells in vivo or by the ectopically or stress-induced overexpressed legumain in model systems in vitro.

EXPERIMENTAL PROCEDURES

Materials. DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), androstan, nonessential amino acids, sodium pyruvate, insulin, and penicillin−streptomycin−nystatin were obtained from Biological Industries, Israel; EGM-2 MV medium was obtained from Cambrex; Neomycin (G418) was obtained from Chemicon International; XTT reagent was obtained from biological industries; rhlegumain was obtained from R&D systems, USA; ketamine was obtained from Ketaset, Canada; xylazin was obtained from Medical Market; etoposide, polybrene, *n*-octyl β-D-glucopyranoside, protease inhibitor cocktail (P2714−1BTL), and CHAPS were obtained from Sigma Aldrich, Israel. CBZ-Ala-Ala-Asn-AMC (compound **1**) was obtained from Bachem. CBZ-Ala-Ala-Asn(Trt)-OH (compound **2**) was obtained from Chinese Peptide Company. Bicinchoninic acid (BCA) kit and enhanced chemiluminescence kit (SuperSignal) were obtained from Pierce, USA. Nitrocellulose membrane was obtained from Whatman, Germany. Endothelial mitogen was obtained from Biomedical Technologies, Stoughton, USA. Heparin was obtained from Laboratoire Choay, Paris, France. Monoclonal mouse antiactin was obtained from Sigma (A 1978); goat antimouse immunoglobulin G (IgG) horseradish peroxidase (HRP) was obtained from Jackson Immunoresearch (115-035-166); goat polyclonal antilegumain was obtained from R&D Systems (AF2199); goat polyclonal antilegumain was obtained from Santa Cruz (sc-47105); rabbit antigoat HRP was obtained from Chemicon International (AP106P); rabbit anti-p38 was obtained from Sigma; and goat antirabbit IgG HRP was obtained from Jackson Immunoresearch (11-035-144). The pcDNA3/legumain plasmid was kindly provided by David Roodman and Chang-Sook Hong (University of Texas Health Science Center, San-Antonio, Texas, USA).

Cell Culture. 293 HEK cells were kindly provided by Professor Sara Lavi (Tel Aviv University, Tel Aviv, Israel); U87-human glioblastoma and PC3-human prostate adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA; TRAMP C2 cells were kindly provided by Professor Norman Greenberg (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA.); CT26 murine colon carcinoma cells were kindly provided by Professor Eliezer Flescher (Tel Aviv University, Tel Aviv, Israel); human umbilical vein endothelial cells (HUVEC) were isolated in our laboratory as previously described (*29*). Umbilical cords were collected at Lis Maternity Hospital, Sourasky Medical Center, Tel Aviv, Israel. This was approved by the Institutional Review Board (IRB). CT26 colon carcinoma, 293 HEK, PC3-human prostate adenocarcinoma cells were cultured

in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, and 2 mM L-glutamine. TRAMP-C2: cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, 15 un/mL insulin, nonessential amino acids X 1, 10 μM androstan, 1 mM sodium pyruvate, and 2 mM L-glutamine. 293 HEK-Leg and U87 human glioblastoma cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, and 2 mM L-glutamine. For selection purposes, 200 μg/mL of G418 was added. HUVEC: Cells were cultured in M-199 supplemented with with 20% FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin and 2 mM L-glutamine, 50 μg/mL endothelial mitogen, and 5 U/mL heparin. All cells were grown at 37 °C; 5% CO_2.

Prodrug Synthesis. *Compound* **3.** Compound **2** was dissolved in dry THF (1 g, 1.53 mmol), and the mixture was cooled to −15 °C. *N*-Methyl morpholine (170 μL, 1.53 mmol) was added, and then isobutylchloroformate (239 μL, 1.843 mmol) was added dropwise. The reaction was stirred for 15 min, then, the commercially available *N*-Boc,*N*-methyl ethylenediamine (401.4 mg, 2.30 mmol) was added. The reaction was allowed to warm to room temperature, was stirred for 1 h, and was monitored by TLC (EtOAc/hexane, 85:15). Upon completion of the reaction, the solvent was removed under reduced pressure, and the crude product was purified by using column chromatography on silica gel (EtOAc/hexane, 85:15) to give compound **3** (808 mg, 65%) as a white solid.

^{1}H NMR (400 MHz, MeOD): $\delta = 7.33-7.14$ (20H, m); 5.09 (2H, bs); 4.67 (1H, m); 4.14−4.09 (2H, m); 3.45−3.25 (4H, bs); 3.08 (1H, dd); 2.84 (3H, s); 2.58 (1H, dd); 1.43 (9H, s); 1.34 (6H, s). ^{13}C NMR (100 MHz, MeOD): $\delta = 174.96$, 174.34, 173.74, 172.66, 158.41, 158.11, 146.16, 138.20, 130.43, 81.57, 68.85, 68.85, 52.50, 50.20, 40.39, 39.56, 37.21, 20.20, 19.33, 18.99.

Compound **4.** Compound **3** (100 mg, 0.123 mmol) was dissolved in 1 mL of TFA and stirred for 10 min. The excess of acid was removed under reduced pressure, and the crude amine salt was dissolved in 1 mL of DMF. The activated carbonate of etoposide (*30*) (111 mg, 0.147 mmol) was added, followed by the addition of triethylamine (163 μL, 1.17 mmol). The reaction was stirred for 30 min and was monitored by TLC (EtOAc/MeOH 90:10). Upon completion of the reaction, the solvent was removed under reduced pressure, and the crude product was purified by using column chromatography on silica gel (EtOAc/MeOH 90:10) to give compound **4** (98 mg, 75%) as a white powder.

^{1}H NMR (400 MHz, MeOD): $\delta = 7.33$ (5H, bs); 6.97 (1H, s); 6.49 (1H, s); 6.30 (2H, s); 5.92 (2H, s); 5.06 (2H, m); 4.95 (1H, m); 4.80 (1H, m); 4.66 (2H, m); 4.30 (2H, m); 3.63 (6H, s); 3.50 (7H, m); 2.94 (4H, m); 2.67 (3H, m); 1.32−1.2 (9H, m). ^{13}C NMR (100 MHz, MeOD): $\delta = 177.97$, 176.93, 173.88, 159.09,156.59, 155.58,153.83, 150.53, 148.98, 140.29, 134.28, 130.93,130.01, 129.81, 129.41, 111.72, 111.42, 109.41, 109.28, 103.79, 103.44, 101.25, 82.22, 76.35, 74.98, 74.49, 70.22, 69.61, 68.36, 68.05, 57.10, 52.92, 49.84, 48.88, 48.54, 45.77, 42.69, 39.75, 37.83, 21.12, 18.43, 17.66. HRMS (MALDI-TOF) calculated for $C_{51}H_{62}N_6O_{20}$ 1101.3911 [M + Na^+], found 1101.3899.

Evaluation of the Activity of Rhlegumain and Endogenous Legumain on Its Commercial Substrate CBZ-Ala-Ala-Asn-AMC. Prior to all activity assays, rhlegumain (R&D, Lot MVY01651) was activated by incubation at a concentration of 100 μg/mL in 50 mM NaOAc and 0.1 M NaCl, pH 4.0, for 2 h at 37 °C.

The specific activity of rhlegumain was measured using 100 μM of rhlegumain's commercial substrate CBZ-Ala-Ala-Asn-AMC and 50 ng of active enzyme in 100 μL of 50 mM MES and 0.25 M NaCl at pH 5.0. Cleavage of CBZ-Ala-Ala-Asn-AMC was measured at excitation $\lambda = 380$ nm and emission $\lambda = 460$ nm, in a fluorescence plate reader.

In order to measure the activity of the endogenous cellular legumain, confluent cells were first harvested in PBS and centrifuged at 3,000 rpm for 3 min at 4 °C. The pellet was resuspended in lysis buffer (40 mM citric acid, 121 mM $NaHPO_4$, 1 mM EDTA, and 1% w/v *n*-octyl-β-D-glucopyranoside, pH 5.8) and incubated on ice for 10 min with occasional vortexing. Three cycles of freezing and thawing were performed. Cells were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was collected and tested for its protein concentration using the BCA kit. Then, the enzymatic activity of endogenous legumain was measured using 5−10 μL of cell lysate (depending on total protein concentration), which were placed in a 96-well microtiter plate. The reaction was initiated by the addition of 190 μL of CBZ-Ala-Ala-Asn-AMC solution in assay buffer (40 mM citric acid and 121 mM $NaHPO_4$, pH 5.8, containing 1 mM dithiothreitol, 1 mM EDTA, and 0.1% CHAPS) to give a final concentration of 10 μM substrate. The plates were incubated at 25 °C, and the level of legumain activity was calculated from the obtained cleaved substrate. Fluorescence levels of the released AMC were measured at excitation $\lambda = 360$ nm and emission $\lambda = 460$ nm, in a fluorescence plate reader.

HPLC Analysis. To test etoposide prodrug cleavage by rhlegumain, 100 μL (50 mM MES and 0.25 M NaCl, pH 5.0) of 500 μM prodrug was incubated with 250 ng of activated rhlegumain overnight at 37 °C. A control tube with 100 μL of 500 μM prodrug without rhlegumain was incubated under the same conditions. HPLC analysis was performed using a C-18 RP (reverse phase) analytical column; $\lambda = 290$ nm; flow, 1 mL min^{-1}; eluent, ACN/H_2O; gradient program, $t = 0$ (10% ACN/90% H_2O)−$t = 30$ (100% ACN/0% H_2O).

To test etoposide prodrug cleavage by cell lysates, 293 HEK-Leg, 293 HEK, and CT26 cells were lysed according to cell lysis (endogenous legumain activity assay) described in this section. The etoposide prodrug (250 μM) was incubated with 75 μL of cell lysate (giving total volume of 200 μL) at 37 °C. HPLC analysis was performed using a C-18 RP analytical column; $\lambda = 290$ nm; flow, 1 mL min^{-1}; eluent, ACN/H_2O; gradient program, $t = 0$ (10% ACN/90% H_2O)−$t = 30$ (100% ACN/0% H_2O).

Stable Transfection of Legumain into 293 HEK Cells. 293 HEK cells were plated a day before tranfection to reach 80% confluency during transfection. Cells were stably transfected with pcDNA3/legumain by calcium phosphate/DNA precipitation (*31*). Transfected cells were cultured in medium containing 1.2 mg/mL of G418. Every 3 days, the selection media containing G418 was changed. Following 2 weeks of culture in selection media, cell lysates and conditioned medium were tested for legumain expression by Western blot.

Cell Starvation. CT26 murine colon carcinoma, 293 HEK, TRAMP C2, PC3 human prostate adenocarcinoma, and U87 human glioblastoma cells were plated to reach 90% confluence at starvation. The medium of each cell line was replaced with serum-free medium. An additional plate of each cell line was left with cultured medium as the control. All plates were further incubated at 37 °C and 5% CO_2 for 6 h. Cells were harvested and kept in −80 °C.

Western Blot Analysis. Prior to Western blot analysis, the medium was concentrated and lyophilized O.N. (Benchtop-K, VirTis). The powder was resuspended in 1 mL of DDW.

For cell lysate analysis by Western blot, confluent cells were harvested with PBS and centrifuged at 3,000 rpm for 3 min at 4 °C. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% triton,

supplemented with 1% protease inhibitor cocktail) and incubated on ice for 20 min with occasional vortex. Cells were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was collected and tested for its protein concentration by the BCA kit.

For tumor and organ analysis by Western blot, murine tumors and organs were weighed. Lysis buffer (50 mM sodium citrate, pH 5.8, 0.1 M NaCl, 1 mM EDTA, and 2 mM β-mercaptoethanol) was added in 8 parts (v/w). Tissues were homogenized using a homogenizer (Politron, Kinematika, Switzerland), and three cycles of freezing and thawing were performed. The homogenates were centrifuged at 14,000 rpm at 4 °C for 20 min. The supernatant was collected and tested for its protein concentration using the BCA kit.

All cell lysates, tissue lysates, and cell medium were boiled in sample buffer × 2 and loaded onto a 12% PAGE gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes, which were blocked with nonfat milk. For legumain detection, goat antilegumain was incubated with the membrane O.N. (1:1,000 dilution with R&D or 1:200 with Santa Cruz, depending on the samples). The membrane was washed three times with TBST and then incubated with HRP-labeled antigoat IgG (1:5,000) for 1 h. For p38 detection, rabbit anti-p38 was incubated with the membrane for 2 h (1:10,000 dilution). The membrane was washed three times with TBST and incubated with HRP labeled antirabbit IgG (1:10,000) for 1 h. Following incubation with the antibodies, membranes were washed three times with TBST, incubated with Supersignal kit, exposed to film, and developed.

Cell Proliferation Assay. 293 HEK or 293 HEK-Leg cells were plated at 4000 cells/well in a 96-well plate (37 °C; 5% CO_2). For serum-starved cell lines, medium was replaced with serum-free DMEM for 6 h. Cells were then challenged with etoposide, etoposide prodrug, or etoposide prodrug preincubated O.N. with rhlegumain, at serial concentrations. Control cells were grown with DMEM with growth factors and serum. Following 72 h, cell growth was evaluated by an XTT assay (*32*).

For HUVEC proliferation assay, cells were plated at 15,000 cells/well (37 °C; 5% CO_2). Following 24 h, cells were challenged with etoposide, etoposide prodrug, etoposide prodrug preincubated O.N. with rhlegumain, at serial concentrations, Control cells were grown with EGM2-MV. Following 72 h, HUVEC were counted with a Z1-Coulter particle counter (Beckman Coulter).

Animal Studies. All animal procedures were performed in compliance with Tel Aviv University, Sackler School of Medicine guidelines and protocols approved by the Institutional Animal Care and Use Committee. Animal were treated as follows: (a) 7 week old male Balb/c mice were inoculated s.c. with 1×10^6 CT26 cells. Upon exceeding 1000 mm^3 in volume, mice were euthanized by cervical dislocation, and immediately, all tumors and organs were removed and kept at −80 °C. Tumor volume was measured with a caliper and calculated according to the formula width × length^2 × 0.52. (b) 7 weeks old male C57/BL6 mice were inoculated orthotopically into the prostate with 1×10^6 TRAMP C2 cells. Following 2 weeks, mice were euthanized by cervical dislocation, and immediately, all tumors and organs were resected and kept at −80 °C.

Semiquantitative RT-PCR. Total RNA extraction was performed using the EZ-RNA kit (Biological Industries) according to the manufacturer's instructions. The reverse transcriptase reaction was performed with the EZ-first Strand cDNA Synthesis kit (Biological Industries) using oligo(dT) primers. The PCR reactions were carried out using PCR-ReadyTM High Specificity (Syntezza) in an ATC 401 thermocycler (Apollo, CLP). To get semiquantitative results, the number of cycles for each reaction was kept at a minimum.

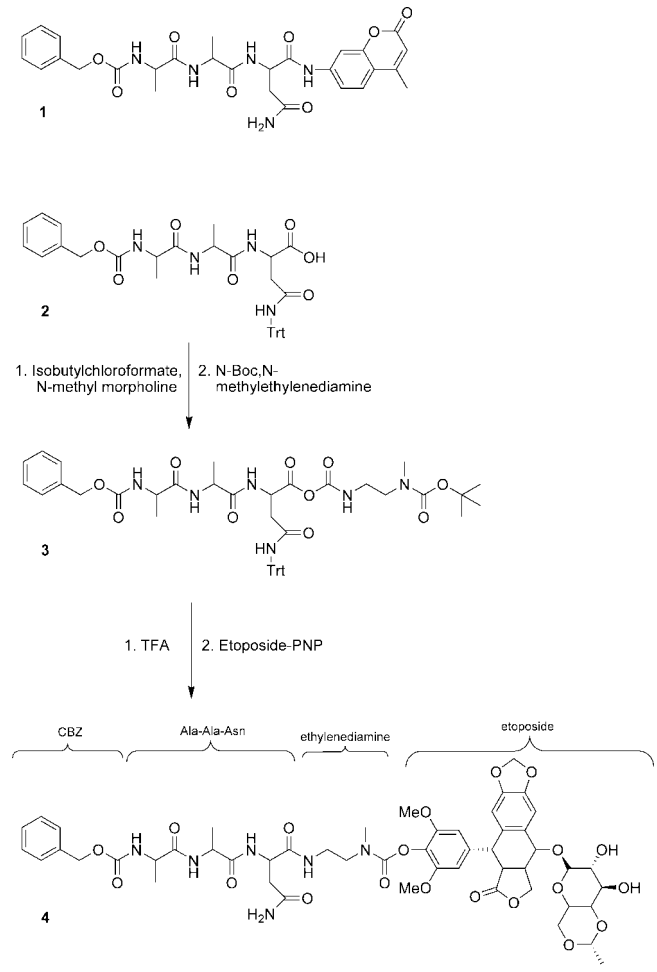

Figure 1. Synthesis of etoposide prodrug (compound **4**). Etoposide was conjugated to the tripeptide (legumain substrate) through an amine spacer (*N*-Boc,*N*-methyl ethylenediamine) to give the final prodrug-compound **4.**

PCR primers: Legumain, 5′-AAGCACTGGGTGGTGATTG-3′ and 5′-TGAGGTCAAGGT GTGTGA-3′; GAPDH, 5′-CCATCACCATCTTCCAGGAGC-3′ and 5′-GGCATGGACT-GTGGTCATGAG-3′.

RESULTS

Etoposide Prodrug Synthesis. On the basis of the standard substrate of legumain, we synthesized a tripeptide substrate, linked to an amine spacer (which is used for etoposide conjugation). The synthesis of the etoposide prodrug is presented in Figure 1. Compound **2**, CBZ-Ala-Ala-Asn(Trt)-OH, was coupled with the commercially available, *N*-Boc,*N*-methylethylenediamine, yielding compound **3**. Compound **3** was dissolved in TFA for deprotection of both the Trityl and the Boc protecting groups. Then, the carbonate derivative of etoposide was reacted with the deprotected substrate, generating the final prodrug **4** (Figure 1).

rhLegumain Cleaved Its Substrate CBZ-Ala-Ala-Asn-AMC More Potently at pH 6.5 than at pH 5.0. Enzymatic activity of legumain was first evaluated by incubating rhlegumain with the commercially available substrate CBZ-Ala-Ala-Asn-AMC. Unexpectedly, the enzymatic activity was greater at a pH value of 6.5 rather than at 5.0. No enzymatic activity was noted at pH 7.0 due to the fact that legumain undergoes denaturation at these basic conditions. The substrate alone showed no spontaneous release of AMC, when incubated without rhlegumain (Figure 2a).

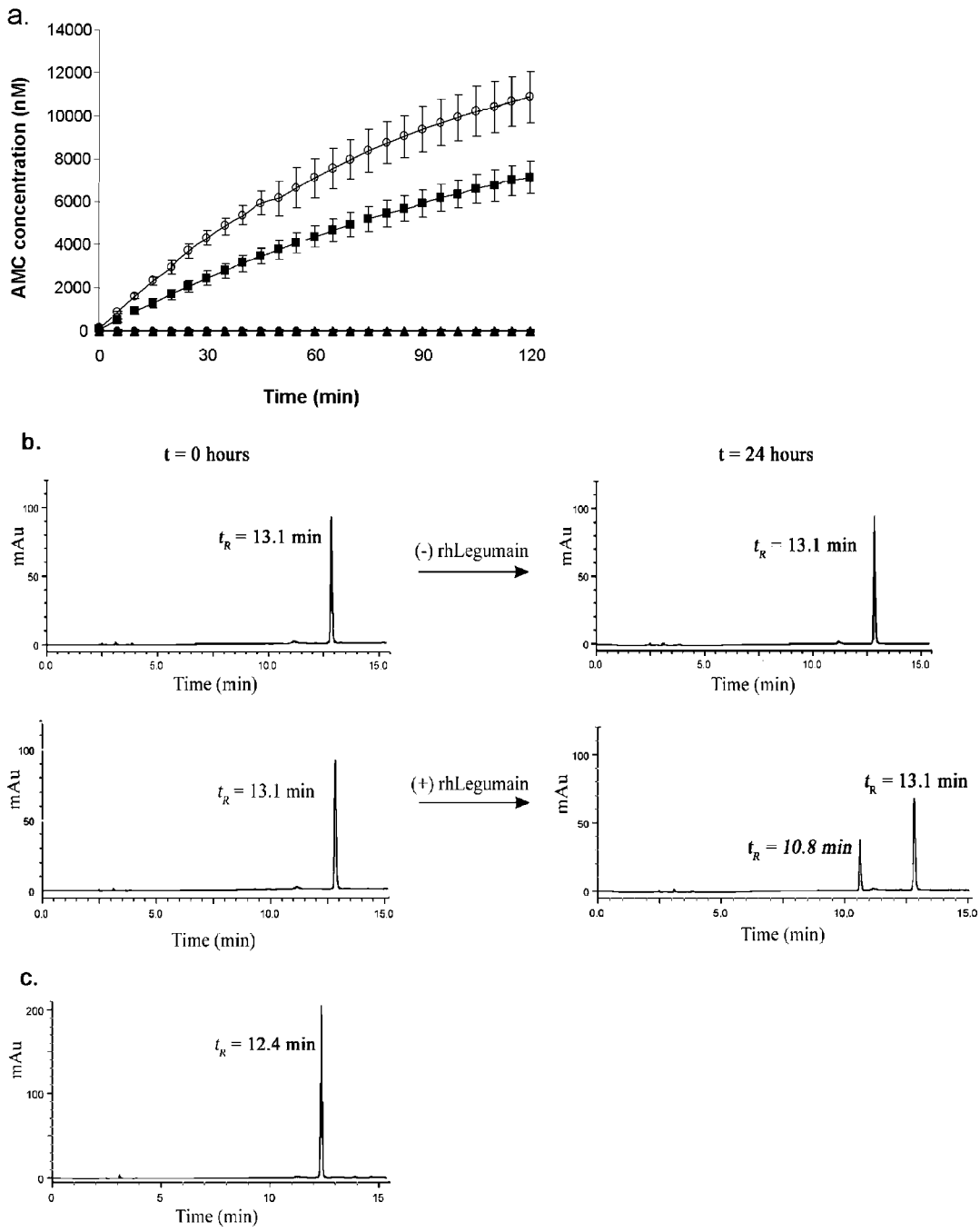

Figure 2. Active rhlegumain potently cleaves its substrate CBZ-Ala-Ala-Asn-AMC and the etoposide prodrug. (a) AMC release at pH 5.0 (■), pH 6.5 (○), pH 7.0 (♦), or in the absence of rhlegumain (▲). Data represent the mean ± SD (b) HPLC analysis of etoposide prodrug in the absence or presence of rhlegumain at $t = 0$ and $t = 24$ h. (c) HPLC analysis of free etoposide.

Characterization of the Etoposide Prodrug. In order to establish its capability to be cleaved by legumain, the etoposide prodrug was incubated with rhlegumain and evaluated using HPLC. The etoposide prodrug eluted as a single peak with an average retention time of $t_R = 13.1$ min and exhibited no self-degradation for 24 h. Incubation of the prodrug with rhlegumain for 24 h resulted in 33% of an inactive etoposide−ethylenediamine (Figure 2b). Further basic conditions were supplied in order for the linker to be released and free active etoposide to be generated, as previously described (*33*). Under the tested conditions, the ethylenediamine failed to undergo cyclization and release free etoposide. Free etoposide eluted as a single peak with $t_R = 12.4$ min (Figure 2c).

293 HEK-Leg Express Legumain. It has been previously reported that legumain expression in vitro is scarce (*19*). Therefore, 293 HEK cells were stably transfected to constitutively overexpress legumain. Legumain expression was evaluated by Western blot analysis. Commercial active (46 kDa) and inactive (56 kDa) rhlegumain were used as size reference controls (Figure 3a). Active legumain was detected in 293 HEK-Leg cells as 46 and 36 kDa bands (mature and nonmature protein) and inactive legumain as a 56 kDa band. 293 HEK-Leg cells also secreted legumain to the conditioned medium. No signal was found in the nontransfected 293 HEK cell lysate and medium. p38 was used as the loading control (Figure 3b).

Expression of Legumain Was Elevated due to Serum Starvation. As previously described, the tumor microenvironment is characterized by a defective supply of blood and nutrients, which exposes the tumor cells to hypoxia and starvation.

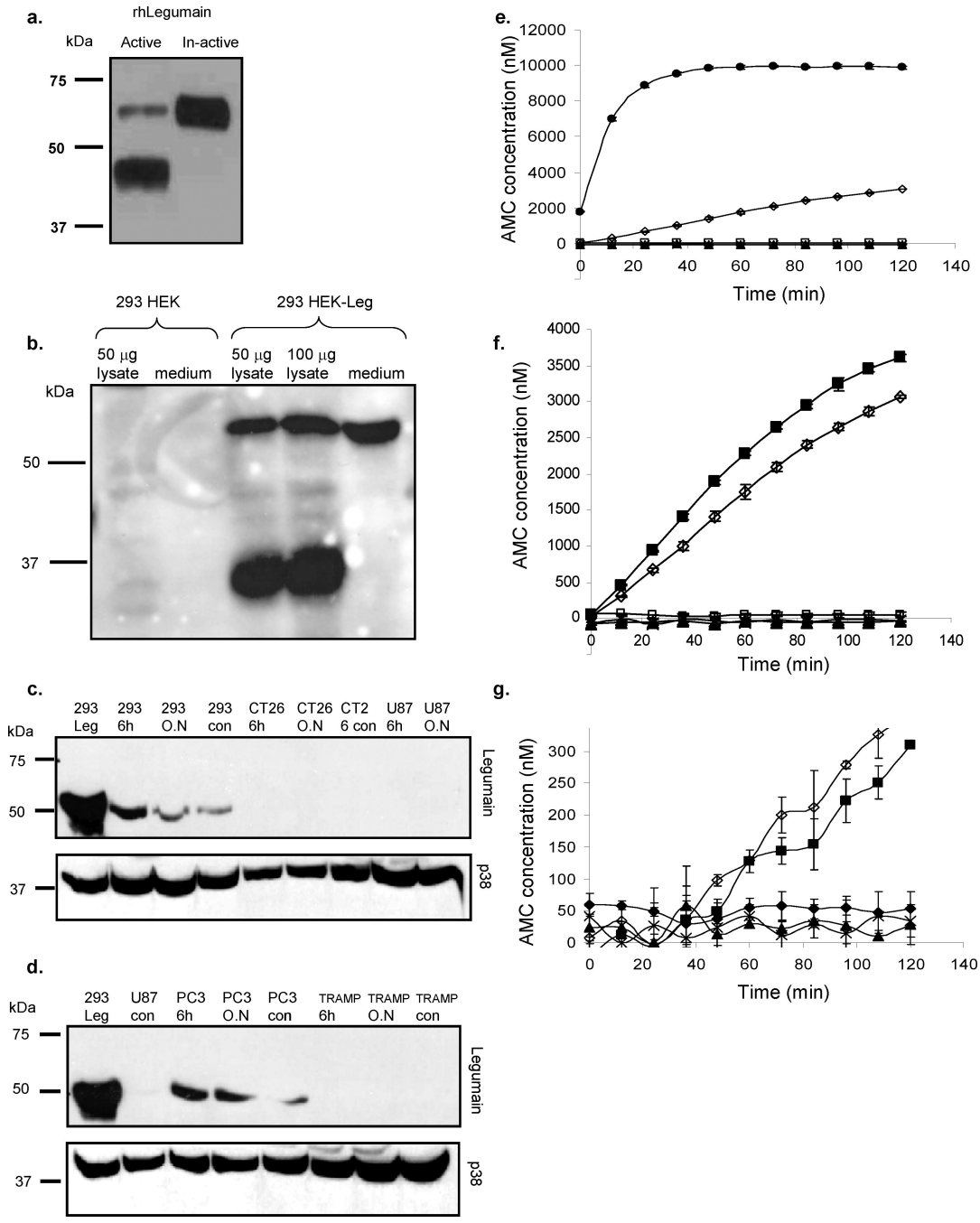

Figure 3. Legumain expression pattern and activity. (a) Western blot analysis of active (46 kDa) and inactive (56 kDa) rhlegumain. (b) Western blot analysis of cell lysate and conditioned medium of 293 HEK and 293 HEK-Leg cells. Active legumain is detected at 46 and 36 kDa (nonmature and mature forms, respectively), and inactive legumain is detected as a band of 56 kDa. p38 antibody was used as loading control. (c,d) 293 HEK, CT26 colon carcinoma, U87-human glioblastoma, PC3-human prostate adenocarcinoma, and TRAMP C2 murine prostate adenocarcinoma cells were serum starved for 6 h, O.N., or not starved at all, respectively. Cells were then harvested, lysed, and analyzed by Western blot. (e) Legumain commercial substrate was incubated with 293 HEK-Leg (●) or 293 HEK cell lysate (◊). Substrate alone (□), 293 HEK cell lysate (▲), and 293 HEKLeg cell lysate (×) were controls. A legumain activity assay was performed as described in Experimental Procedures. (f) Control (◊) or serum-starved (■) 293 HEK cell lysates were incubated with substrate. Substrate alone (□) and 293 HEK cell lysate alone, either serum starved (×) or nonstarved (▲) were controls. A legumain activity assay was performed. Data represent the mean ± SD. (g) Control (◊) or serum-starved (▲) PC3 cell lysate were incubated with the substrate. Substrate alone (♦) and PC3 cell lysate alone, either starved (×) or nonstraved (■) were controls. A legumain activity assay was performed. Data represent the mean ± SD.

Therefore, we wanted to determine whether endogenous legumain elevates as a result of serum starvation. Different cell lines were serum-starved and examined for their legumain expression. Only 293 HEK and PC3 prostate adenocarcinoma cell lines expressed legumain in vitro, and the levels of its inactive form increased following serum starvation. p38 antibody was used as the loading control (Figure 3c,d).

Overexpressed Legumain in 293 HEK-Leg Cells Potently Cleaved CBZ-Ala-Ala-Asn-AMC. 293 HEK-Leg cells were found to overexpress the active form of legumain (Figure 3b). Therefore, we evaluated the enzymatic activity of endogenous and overexpressed legumain. 293 HEK-Leg cell lysates were incubated with legumain's commercial substrate CBZ-Ala-Ala-Asn-AMC and released high levels of AMC. 293

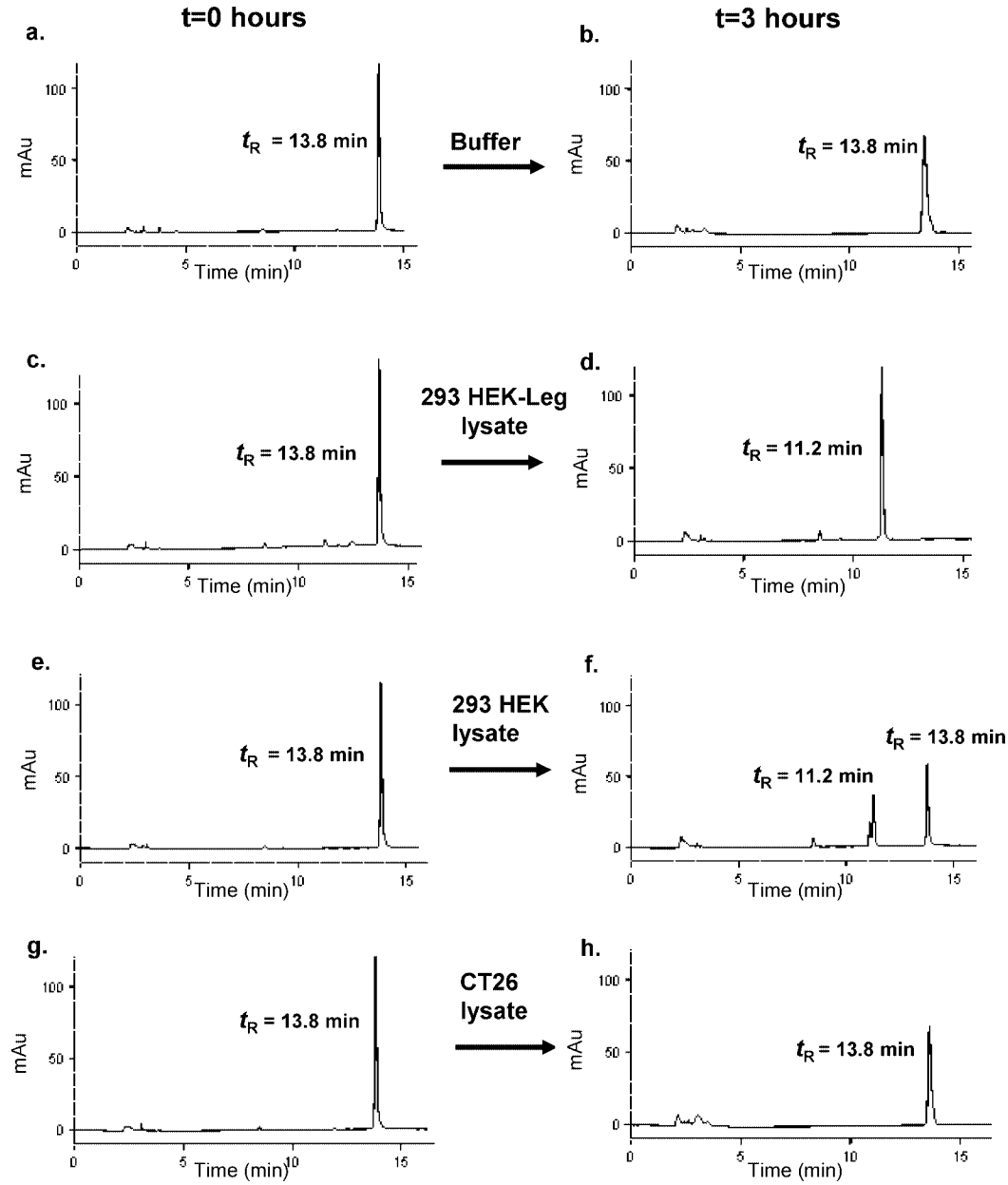

Figure 4. Both endogenous and exogenous legumain effectively and selectively cleave the etoposide prodrug as evaluated by HPLC analysis. (a,b) Etoposide prodrug in buffer at $t = 0$ and 3 h following incubation in buffer. (c,d) Etoposide prodrug with 293 HEK-Leg cell lysate at $t = 0-3$ h. (e,f) Etoposide prodrug with 293 HEK cell lysate at $t = 0-3$ h. (g,h) Etoposide prodrug with CT26 cell lysate at $t = 0-3$ h.

HEK cell lysates showed only a minor enzymatic activity releasing very low levels of AMC (Figure 3e). We further explored whether the elevation in expression of legumain due to serum starvation correlates with an increase in its enzymatic activity. 293 HEK serum-starved cells showed a slight 1.2-fold increase in enzymatic activity toward CBZ-Ala-Ala-Asn-AMC compared to that of nonstarved 293 HEK cells (Figure 3f), while PC3 serum-starved or nonstarved cells showed no significant difference in enzymatic activity (Figure 3g). Substrate alone and cell lysates of starved and nonstarved cells were used as controls.

Overexpressed Legumain Potently and Selectively Cleaved the Etoposide Prodrug. In order to examine whether ectopically expressed legumain specifically cleaves the etoposide prodrug, the prodrug was incubated separately with 293 HEK-Leg, 293 HEK, and CT26 cell lysates. Cleavage was evaluated using HPLC analysis. While the etoposide prodrug alone did not undergo self-hydrolysis within 3 h and elute as a single pick with $t_R = 13.8$ min (Figure 4a,b), incubation of 3 h with 293 HEK-Leg cell lysates resulted in 100% cleavage, generating etoposide−ethylenediamime (etoposide-linker) with $t_R = 11.2$ min (Figure 4c,d). 293 HEK cell lysates, which were previously found to scarcely express legumain, resulted in only 33% cleavage (Figure 4e,f), and CT26 cell lysates left the etoposide prodrug 100% intact following 3 h of incubation (Figure 4g,h).

Etoposide and Etoposide Prodrug Inhibited the Proliferation of 293 HEK and 293 HEK-Leg Cells. To determine the in vitro activity of etoposide and the etoposide prodrug, their inhibitory effect was tested on 293 HEK and 293 HEK-Leg cells. Free etoposide inhibited the proliferation of both cell lines with an IC_{50} of 700 nM. Treatment with the etoposide prodrug alone on both 293 HEK-Leg and 293 HEK cells did not reach the IC_{50} at the concentrations tested ($IC_{50} > 100\ \mu M$). However, when cleaved externally with rhlegumain prior to the proliferation assay, the etoposide prodrug inhibited 293 HEK and 293 HEK-Leg cells with an IC_{50} of 4 μM (Figure 5a).

Serum-Starvation-Induced Inhibition of the Proliferation of 293 HEK Cells by the Legumain-Activated Pro-

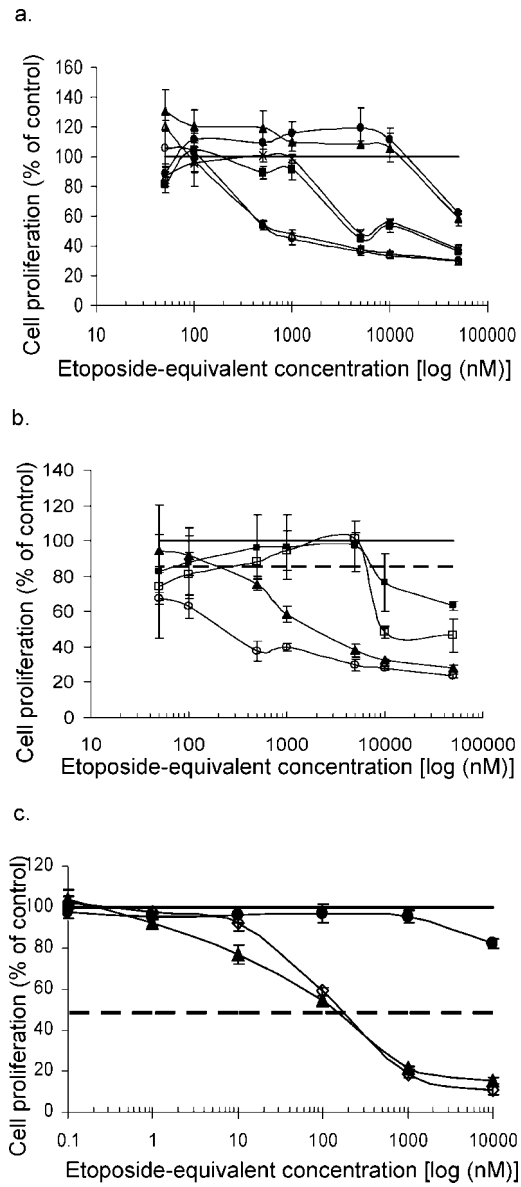

Figure 5. Etoposide and the etoposide prodrug inhibit cell proliferation in vitro. (a) Proliferation assay of 293 HEK-Leg (△,▲) and 293 HEK cells (○,●), following treatment with free etoposide and etoposide prodrug, respectively. 293 HEK cells were also assayed for proliferation following treatment with the externally cleaved etoposide prodrug (■). (b) Proliferation assay of control (black triangles and squares) and serum-starved (white circles and squares) 293 HEK cells, following treatment with free etoposide and etoposide prodrug, respectively. (c) HUVEC proliferation assay following incubation with free etoposide (◊), the etoposide prodrug (●), and the legumain-activated etoposide prodrug (▲). Data represent the mean ± SD.

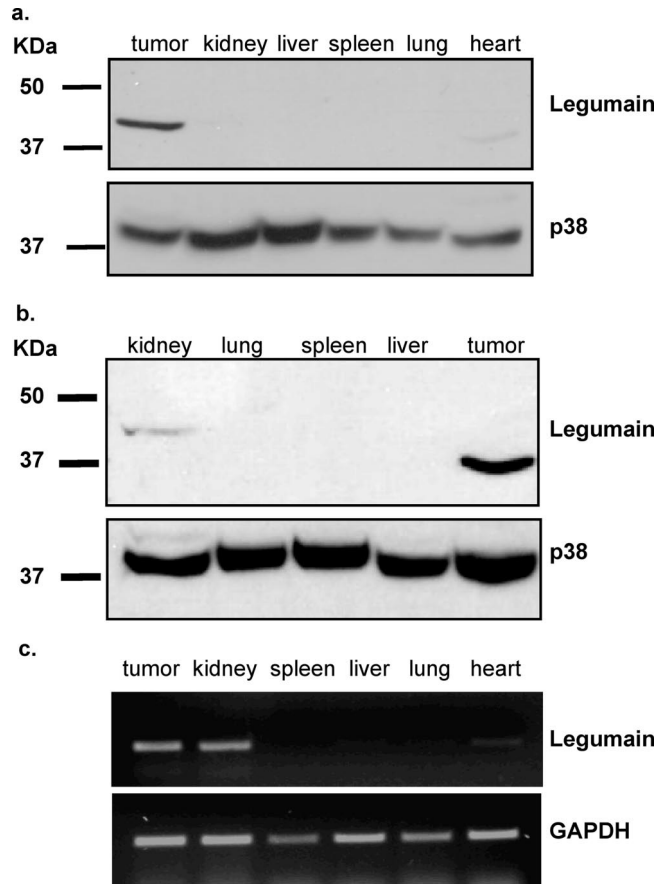

Figure 6. Legumain is overexpressed in murine tumors inoculated in mice compared to normal organs. (a) CT26 tumors inoculated s.c. in Balb/c mice and their normal organs were dissected, homogenized, lysed, and analyzed for legumain expression by Western blot. p38 antibody was used as the loading control. (b) Orthotopic TRAMP-C2 prostate tumors inoculated in C57/BL6 and their normal organs were dissected, homogenized, lysed, and analyzed for legumain expression by Western blot. p38 antibody was used as the loading control. (c) Orthotopic TRAMP-C2 prostate tumors inoculated in C57/BL6 and their normal organs were dissected and homogenized, and RNA was isolated. Legumain mRNA levels were monitored and calibrated to GAPDH mRNA levels (semiquantitive RT-PCR).

drug at High Concentrations. To determine whether the potency of the etoposide prodrug elevates because of the increase in legumain expression, the inhibitory effect of etoposide and the etoposide prodrug was tested on 293 HEK serum-starved or nonstarved cells. Serum starvation of 293 HEK cells was performed in order to elevate legumain expression. Free etoposide inhibited proliferation of both starved and nonstarved cells with IC_{50} values of 200 nM and 2 μM, respectively. The prodrug also had a more toxic effect on serum-starved cells, but only at concentrations higher than 5 μM. At lower concentrations, the prodrug had the same effect on cells grown in the presence or absence of serum (Figure 5b).

Etoposide and the Etoposide Prodrug Inhibited HUVEC Proliferation. It has been recently shown that legumain is also overexpressed in tumor endothelial cells in vivo (*18*). Hence, having shown the inhibitory effect of etoposide on 293 HEK cells, its ability to inhibit HUVEC proliferation was evaluated. Free etoposide and the legumain-activated etoposide prodrug (cleaved externally with rhlegumain prior to the proliferation assay) inhibited HUVEC proliferation with an IC_{50} of 150 nM (Figure 5c). The prodrug alone did not have any inhibitory effect on HUVEC proliferation.

Legumain Is Overexpressed in Murine Tumors Inoculated in Mice Compared to Healthy Organs. In order to demonstrate the potential of an etoposide prodrug activated by legumain in vivo, we examined the expression of legumain in murine tumors inoculated in mice and their healthy organs. CT26 derived tumors and organs of Balb/c mice (Figure 6a) and TRAMP-C2 murine prostate adenocarcinoma and organs of C57/BL6 mice (Figure 6b,c) were lysed and evaluated for legumain expression by Western blot and semiquantitive RT-PCR. Legumain is highly expressed in tumor tissues as opposed to other healthy organs. CT26 cells, subcutaneously inoculated in a Balb/c mouse, showed high expression of murine legumain, while only low levels of legumain were detected in the heart, and no legumain at all in the kidney, liver, spleen, or lung lysates (Figure 6a). TRAMP-C2 prostate tumors, orthotopically inoculated in C57/BL6 mice, expressed high levels of legumain, while

the kidney tissue lysate expressed low levels, and in other organs tested no legumain was detected (Figure 6b). Accordingly, legumain mRNA levels were also higher in the tumor and the kidney in comparison to that in other tissues tested (Figure 6c).

DISCUSSION

The pursuit for effective strategies of targeted chemotherapy treatments for various neoplastic pathologies is still ongoing, as many novel compounds are already in clinical trials. A common method for targeting chemotherapeutic agents specifically to the tumor is the design of a prodrug platform that covalently links the drug to a peptide cleavable by a certain overexpressed protease in the tumor and its microenvironment. Such is legumain, which represents a novel candidate target for chemotherapeutic and antiangiogenic prodrug therapy.

To date, there are only two ongoing prodrug preclinical studies based on legumain, one of which covalently links a peptide cleavable by legumain to doxorubicin. Wu et al. showed that *N*-Suc-Ala-Ala-Asn-Leu-doxorubicin, named LEG-3, was cleavable by legumain and released Leu-doxorubicin at the tumor site (*19*). The second prodrug therapy is a legumain-based DNA vaccine, which leads to suppression of tumor growth and metastasis (*21, 22*).

In this study, we described the synthesis of a prodrug that covalently links the chemotherapeutic agent etoposide to legumain's substrate, CBZ-Ala-Ala-Asn-ethylenediamine-etoposide. The prodrug is designed to be cleaved at acidic conditions by legumain, at the asparagine residue bound to ethylenediamine through a carbamate bond, creating an ethylenediamine−etoposide moiety. According to the chemical synthesis technique established by Perry et al. (*33*), the ethylenediamine linker undergoes cyclization at physiological pH, releasing free etoposide. Thus, the prodrug is designed to be cleaved by legumain in the cell lysosome at acidic pH values and undergo cyclization and etoposide release in the cell cytoplasm.

Enzymatic activity of rhlegumain is known to be optimal at pH 5.0. We evaluated its activity at a pH value of 6.5, an acidic level that better reflects the neoplastic environment, in comparison to the supposedly optimal pH 5 and 7 (Figure 2a). Legumain's enzymatic activity was the highest at pH 6.5, further establishing the concept of it being a suitable target for anticancer prodrug therapy. Evaluation of the enzymatic activity of the commercial legumain led to testing its ability to cleave the prodrug using HPLC analysis (Figure 2b). Our results revealed that the prodrug is in fact cleavable by legumain and generates the ethylenediamine−etoposide moiety at acidic conditions.

We have transfected cells to overexpress legumain in vitro (293 HEK-Leg), which was shown to be highly active against CBZ-Ala-Ala-Asn-AMC (Figure 3b,e). We were also interested in exploring whether stress such as serum starvation would elevate legumain expression in vitro in various cell lines. Our interest was based on the notion that legumain is a stress-responsive gene, as previously suggested by Lui et al. (*18*). This idea was not investigated or tested empirically. Thus, we serum starved various cell lines and examined their endogenous legumain expression (Figure 3d,e). Our findings revealed that legumain expression, though in its inactive form of 56 kDa, is elevated in both 293 HEK and PC3 cell lines following 6 h of serum starvation. Overnight serum starvation did not further elevate legumain expression, probably due to a decrease in cell metabolism, caused by the prolonged stress. The present study is the first to show an increase in legumain expression due to serum starvation in vitro, in any kind of cell line. Although the level of legumain expression was raised, only a slight increase in the enzymatic activity was detected in the serum-starved versus the control 293 HEK cells. There was no difference between the level of legumain enzymatic activity in the control or serum-starved PC3 cells, when tested on the commercial substrate of legumain. This is probably due to the fact that upon serum starvation, only the inactive form of legumain expression level was augmented (Figure 3c,d).

293 HEK-Leg cells cleaved 100% of the prodrug, whereas 293 HEK cells, which express low levels of legumain, cleaved only 33% of it. CT26 cell lysate, in which no legumain expression was found, similarly to our working buffer alone, did not cleave the prodrug at all (Figure 4). This result indicates not only that our transfected 293 HEK-Leg cells successfully cleaved the prodrug but also that the prodrug is specifically and exclusively cleaved by legumain and not by other proteases, as demonstrated by the CT26 cell line, which does not express legumain. Furthermore, the prodrug remains stable and does not undergo nonspecific self-hydrolysis within 72 h.

Establishment of the specificity of the prodrug to be cleaved by legumain enabled us to test its cytotoxic potential. We evaluated the inhibitory effect of the prodrug on the proliferation of 293 HEK and 293 HEK-Leg cells (Figure 5a). As expected, the prodrug did not inhibit the proliferation of 293 HEK cells, and free etoposide inhibited the proliferation of both 293 HEK and 293 HEK-Leg in the same manner, achieving similar IC_{50} values. In contrast to our expectation, the prodrug failed to inhibit the proliferation of 293 HEK-Leg cells. This disappointing result might be due to the fact that upon cleavage by legumain at acidic conditions, the ethylenediamine−etoposide either failed to exit the lysosome and reach the cytoplasm or that cyclization did not occur. Another possibility that can explain the failure of the prodrug to inhibit the proliferation of 293 HEK-Leg cells concerned its uptake by the cells. Even though the prodrug is relatively small in its molecular size and is compact in structure, it is possible that the prodrug failed to enter the cells and therefore did not inhibit cell proliferation. Furthermore, it is plausible that the prodrug did internalize into the cell but did not succeed in entering the lysosome in order to be cleaved. A suitable uptake assay on an adequate prodrug pathway examination is necessary and would reveal whether the prodrug internalizes the cell through passive endocytosis, as expected, or whether it is adsorbed around the cell membrane. A future confocal microscopy analysis of a fluorescently labeled prodrug would disclose its subcellular trafficking and localization. Since etoposide is not fluorescent by itself (unlike doxorubicin), it will be necessary to link a suitable fluorescent molecule for detection purposes that does not interfere with the prodrug's cleavage site.

We have demonstrated that expression levels of endogenous legumain elevate in 293 HEK cells following stress. We hypothesized that cells expressing high levels of endogenous legumain may better cleave and activate the prodrug than 293 HEK-Leg cells, which overexpress ectopic legumain. The inhibitory effect of the prodrug on the proliferation of serum-starved 293 HEK cells revealed that serum starvation turned the cells more susceptible to etoposide (Figure 5b). However, serum starvation in itself had no significant inhibitory effect on the proliferation of 293 HEK cells treated with low concentrations of the etoposide prodrug. The influence of serum starvation was noted at concentrations higher than 5 μM etoposide prodrug. The IC_{50} of the etoposide prodrug on serum-starved 293 HEK cells was lower than the IC_{50} of the etoposide prodrug on nonstarved 293 HEK cells. Thus, at relatively high concentrations of the etoposide prodrug, serum starvation increased the prodrug's potency on the inhibition of proliferation of 293 HEK cells by increasing legumain's expression and augmenting the sensitivity of the cells to the treatment.

Legumain has also been reported to be overexpressed in tumor endothelial cells. Therefore, we evaluated the effect of the etoposide prodrug on tumor angiogenesis by examining the

inhibitory potential of etoposide on proliferating endothelial cells (Figure 5c). Our results indicate that etoposide inhibits HUVEC proliferation at concentrations 1 log-lower than tumor cells; hence, tumor endothelial cells would be sensitive to lower doses of etoposide released from the prodrug.

We inoculated two different cell lines in two different strains of mice (CT26 in Balb/c mice and TRAMP C2 in C57BL/6 mice) in order to show that overexpression of legumain is not cell line-dependent or strain-dependent. Western blot and RT-PCR analysis revealed that legumain is in fact overexpressed in tumor tissues rather than in normal tissues, in both cell lines in both strains (Figure 6). Interestingly, although there was no substantial difference between the mRNA levels of legumain in the tumor and kidney tissues from C57/BL6 mice, the legumain protein levels were substantially higher in tumor tissues. This finding suggests that the expression level of legumain is not governed by enhanced transcription and points to the involvement of post-translational events. Further studies should clarify this issue.

In summary, this study describes the synthesis and characterization of an etoposide prodrug designed to be cleaved by the endoprotease legumain. The prodrug was found to be potently and selectively cleaved by all three forms of legumain: the commercial, overexpressed, and endogenous legumain. Therefore, the prodrug should specifically be cleaved at the tumor site, which will warrant this approach as a novel anticancer targeted therapy.

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