Protease-Mediated Fragmentation of *p*-Amidobenzyl Ethers: A New Strategy for the Activation of Anticancer Prodrugs

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A new anticancer prodrug activation strategy based on the 1,6-elimination reaction of *p*-aminobenzyl ethers is described. Model studies were undertaken with the *N*-protected peptide benzyloxycarbonyl-valine-citrulline (Z-val-cit), which was attached to the amino groups of *p*-aminobenzyl ether derivatives of 1-naphthol and *N*-acetylnorephedrine. The amide bond that formed was designed for hydrolysis by cathepsin B, a protease associated with rapidly growing and metastatic carcinomas. Upon treatment with the enzyme, the Z-val-cit-*p*-amidobenzyl ether of 1-naphthol (**2**) underwent peptide bond hydrolysis with the rapid release of 1-naphthol. The aliphatic Z-val-cit-*p*-amidobenzyl ether of *N*-acetylnorephedrine (**5**) also underwent amide bond hydrolysis, but without the ensuing elimination of *N*-acetylnorephedrine. On the basis of these results, the phenolic anticancer drugs etoposide (**6**) and combretastatin A-4 (**7**) were attached to the Z-val-cit-*p*-amidobenzyl alcohol through ether linkages, forming the peptide-drug derivatives **8** and **9**, respectively. Both compounds were stable in aqueous buffers and serum and underwent ether fragmentation upon treatment with cathepsin B, resulting in the release of the parent drugs in chemically unmodified forms. The released drugs were 13−50 times more potent than were the prodrug precursors on a panel of cancer cell lines. In contrast, the corresponding carbonate derivative of combretastatin A-4 (**13**) was unstable in aqueous environments and was as cytotoxic as combretastatin A-4. This result extends the use of the self-immolative *p*-aminobenzyl group for the fragmentation of aromatic ethers and provides a new strategy for anticancer prodrug development.

Introduction

Metastatic carcinomas often express proteolytic enzymes including the cysteine protease cathepsin B,[1−3] matrix metalloproteinases such as collagenases and stromelysins,[4] and serine proteases represented by plasminogen activator and plasmin.[5] These enzymes are thought to be critically involved in the events that lead to metastasis because they are capable of degrading the basement membrane and extracellular matrix around tumor tissue, allowing the tumor cells to migrate and invade into the surrounding stroma and endothelium. Cathepsin B in particular has been shown to be clinically relevant in cancer progression on the basis of studies showing that cytosolic enzyme levels (856 ng cathepsin B/mg of protein, determined from 167 breast cancer patients)were 11 times higher than those in benign breast tissue specimens.[3] Patients with high intratumoral cathepsin B levels suffer a significantly worse prognosis than do patients with low levels.[3] Additional activities associated with these proteases include participation in protease cascades, activation of enzymes and growth factors, and tumor angiogenic stimulation.[1−3]

Several investigators have explored the possibility of exploiting tumor-associated proteases for the development of new cancer chemotherapeutics, which has led to several promising orally active protease inhibitors having both preclinical and clinical antitumor activities.[4] An additional line of research involves the conscription of proteases for anticancer prodrug activation. Toward this end, peptide-containing anticancer prodrugs have been developed that are activated by proteases within solid tumors.[6−19] Several of these agents have led to significant in vitro and in vivo antitumor activities.

There are two general approaches for attaching drugs to peptides for intratumoral proteolytic activation. The drugs can be appended directly to the peptide, leading to prodrugs that can either release the parent drug or a drug that contains vestiges of the bound peptide.[16−18] In the latter case, the released drug may have impaired cytotoxic activity. An additional consideration for direct drug attachment to peptides is the negative influence the drug can have on the kinetics of peptide hydrolysis.

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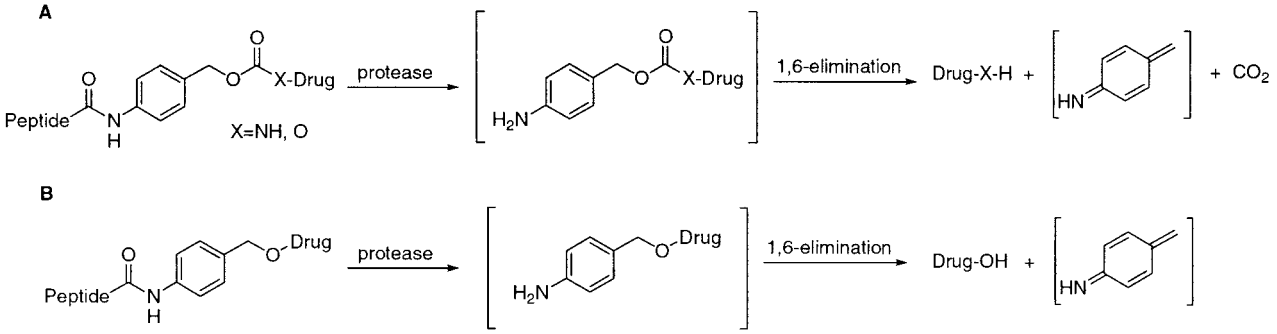
Scheme 1



To circumvent these potential shortcomings, an alternative approach for drug attachment incorporates the use of self-immolative spacers that spatially separate the drug from the site of enzymatic cleavage. The subsequent collapse of the incorporated linkers allows for the elimination of the fully active, chemically unmodified drug from the conjugate upon amide bond hydrolysis. One of the most commonly used spacers is the bifunctional *p*-aminobenzyl alcohol group, which is linked to the peptide through the amine moiety, forming an amide bond. Amine-containing drugs are attached through carbamate functionalities to the benzylic hydroxyl group of the linker. The resulting prodrugs are activated upon protease-mediated cleavage, leading to a 1,6-elimination reaction[20] that releases the unmodified drug, carbon dioxide, and remnants of the linker group (Scheme 1). This methodology, based on the work of Sartorelli, Katzenellenbogen, and co-workers,[21,22] has been applied to the plasmin-catalyzed release of phenylenediamine mustard[8] and anthracyclines[9,12−14] from their corresponding peptide-*p*-amidobenzyl carbamate derivatives and also to the release of doxorubicin and mitomycin C from peptide-*p*-amidobenzyl carbamate peptide derivatives by lysosomal enzymes and cathepsin B.[10,11,15] The same linkage system has also been applied to the activation of anthracyclines in cells that were transfected with carboxypeptidase G2.[23]

The chemistry used for drug attachment has generally been restricted to amine-containing drugs, with the exception of paclitaxel, which was linked through carbonates formed from hydroxyl groups at the 2′- or 7-position.[11,13,14] Unlike many carbonates that are hydrolytically unstable, such paclitaxel 2′- and 7-carbonates were quite stable in aqueous environments, consistent with what had already been reported for other paclitaxel carbonates.[24,25] Many anticancer agents containing reactive hydroxyl groups would not be expected to exhibit such high carbonate stability. For that reason, we explored new methodologies for attaching alcohol-containing anticancer drugs to self-immolative spacers, which would lead to high serum stability and conditional drug release upon peptide bond hydrolysis. Here we describe a novel drug release strategy involving the fragmentation of peptide-*p*-amidobenzyl ether derivatives (Scheme 1). The synthesis, stabilities, fragmentation, and in vitro cytotoxic activities of some representative compounds are described.

Results

The peptide derivative, Z-valine-citrulline-*p*-aminobenzyl alcohol (**1**, Z-val-cit-PAB-OH) has previously been used for the preparation of Z-val-cit-PAB-doxorubicin carbamate, a compound that released active doxorubicin upon treatment with cathepsin B.[10,11] The drug was attached to the peptide through a carbamate linkage, as shown in Scheme 1. To explore the potential of using this drug-elimination pathway for cleaving less labile bonds, ether derivatives of **1** were prepared using either the Mitsunobu reaction to form the naphthol ether **2** or the two-step imidate-substitution reaction to form the *N*-acetylnorephedrine derivative **5** (Scheme 2). These compounds were designed to model anticancer drugs that contain chemically related moieties. HPLC analysis indicated that the naphthol ether **2** was a substrate for bovine spleen cathepsin B and that the products formed were naphthol and Z-val-cit-COOH. The reaction proceeded rapidly (350 nmol/min/mg cathepsin B), and by HPLC, there was no evidence of the build up of the deacylated *p*-aminobenzyl ether. In the absence of added enzyme, there was no breakdown of the starting material after 1 week at 37 °C at pH 5.1 or 7.2 or in pooled human serum (Table 1). These results provide the first indication that *p*-aminobenzyl ethers are capable of undergoing 1,6-elimination reactions. Similar studies undertaken with the *N*-acetylnorephedrine ether **5** demonstrated that the compound was hydrolyzed by cathepsin B, leading to the release of Z-val-cit-COOH as expected. However, no *N*-acetylnorephedrine was detected, suggesting that the *p*-aminobenzyl ether formed after peptide bond cleavage did not undergo further fragmentation. Thus, the nature

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Scheme 2

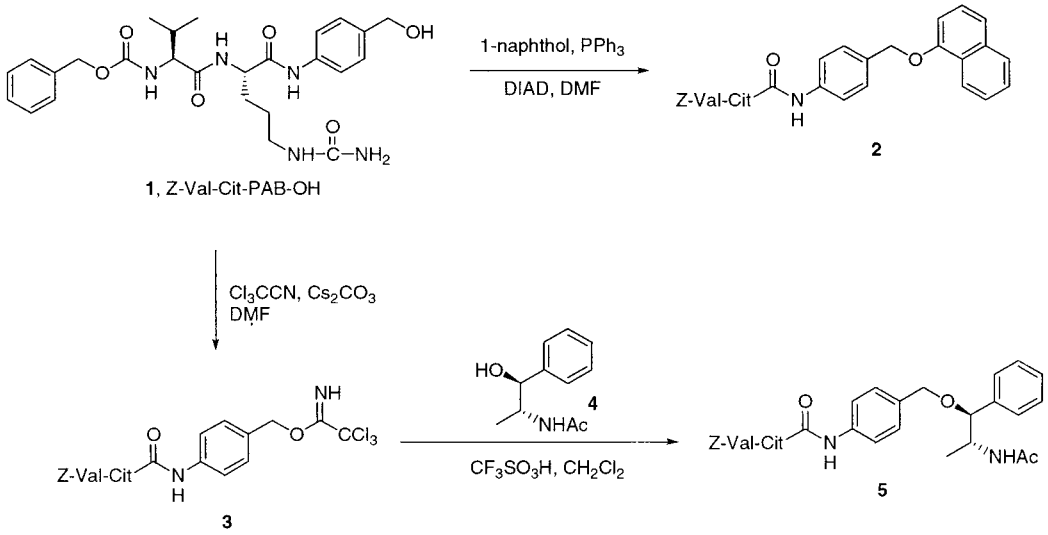
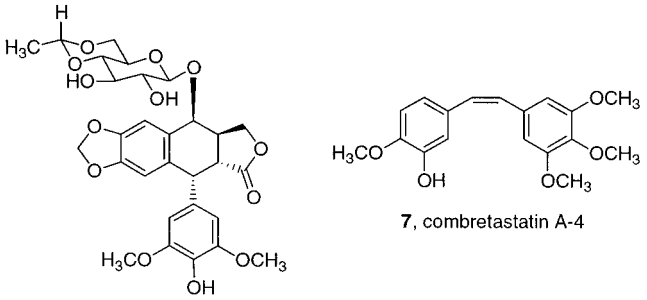


Table 1. Compound Stability in Aqueous Solutions and in the Presence of Cathepsin B

	stability[a]			specific activity cathepsin B[b]
compound	pH 5.1	pH 7.2	human serum	(nmol/min/mg)
2	0% loss 7 d	0% loss 7 d	0% loss 7 d	350
5	0% loss 7 d	0% loss 7 d	0% loss 7 d	145[c]
8	0% loss 7 d	0% loss 7 d	$t_{1/2}$ 48 h[d]	160
9	0% loss 7 d	0% loss 7 d	0% loss 7 d	61
12	$t_{1/2}$ 104 h	$t_{1/2}$ 79 h	$t_{1/2}$ 9 d	150
13	$t_{1/2}$ 62 h	$t_{1/2}$ 55 h	$t_{1/2}$ 45 h	32

[a] Measured as the loss of starting material and the appearance of the released alcohol at 37 °C in phosphate-buffered saline at pH 7.2, acetate buffer at pH 5.1, or pooled human serum. [b] Measured as the loss of starting material at 37 °C in pH 5.1 acetate buffer. [c] Measured as the loss of starting material, which correlated with the appearance of Z-val-cit-COOH. HPLC analysis indicated that *N*-acetylnorephedrine (**4**) was not released upon amide bond hydrolysis. [d] Etoposide (**6**) and the etoposide moiety of **8** were unstable in serum. There was no apparent breakdown of the peptide-linker in **8**. No Z-val-cit-COOH, **1**, or **6** were detected.

Scheme 3



of the leaving group attached to the *p*-aminobenzyl group significantly affects the 1,6-elimination reaction.

On the basis of these results, the anticancer drugs etoposide (**6**) and combretastatin A-4 (**7**) (Scheme 3) were linked to **1** using the coupling conditions shown in Scheme 4. Etoposide is a clinically approved topoisomerase inhibitor that has demonstrated utility in chemotherapeutic combinations for the treatment of leukemia, lymphoma, germ cell tumors, small cell lung tumors, and several other carcinomas.[26] Combretastatin A-4 is a promising antiangiogenic agent that inhibits the polymerization of tubulin.[27] Treatment of **8** and **9** with cathepsin B led to the release of etoposide (**6**) and combretastatin A-4 (**7**), respectively (Table 1). As with the naphthol ether **2**, there was no detectable *p*-aminobenzyl ether, consistent with the fact that peptide hydrolysis is rate-determining in the fragmentation of the aromatic ether bond. Both peptide derivatives were stable at pH 5.1 and 7.2 and in human serum.

For comparison, the carbonate derivatives **12** and **13** were prepared from the activated *p*-nitrophenyl carbonate derivative of *N*-acetylnorephedrine (**4**) and combretastatin A-4 (**7**), respectively (Scheme 5). Both carbonates **12** and **13** proved to be unstable in aqueous environments, in contrast to the corresponding ethers **5** and **9**, respectively (Table 1). As expected, enzymatic hydrolysis of **12** and **13** led to the formation of **4** and **7**. It is noteworthy that there were no significant kinetic differences in cathepsin B-mediated hydrolyses between the peptide-carbonate and peptide-aromatic ether derivatives. Thus, peptide derivatives of *p*-aminobenzyl aromatic ethers are stable in neutral or slightly acidic buffers and undergo facile ether fragmentation upon treatment with an enzyme that cleaves the amide bond.

In vitro cytotoxicity studies were performed on cancer cell lines to determine if the peptide derivatives acted as prodrugs. The cell lines L2987 (human lung adenocarcinoma), WM266/4 (human melanoma), and IGR-39 (human melanoma) were exposed to the agents for 24 h and then washed, and viability was determined 2 days later by measuring the incorporation of ^{3}H-thymidine compared to that in the untreated controls. There were significant differences in the cytotoxic activity of etoposide (**6**) and that of the corresponding peptide ether derivative (**8**) on all three cell lines (Figure 1A−C). At the concentration required for 50% cell kill, etoposide (**6**) was 20−50 times more active than **8**, a result consistent with the loss in cytotoxic activity that has been reported with another phenol derivative of etoposide.[27] Similarly, the combretastatin ether (**9**) was less potent than combretastatin A-4 (**7**) by a factor of 13 on L2987 human lung adenocarcinoma cells (Figure 1D). Significantly, the

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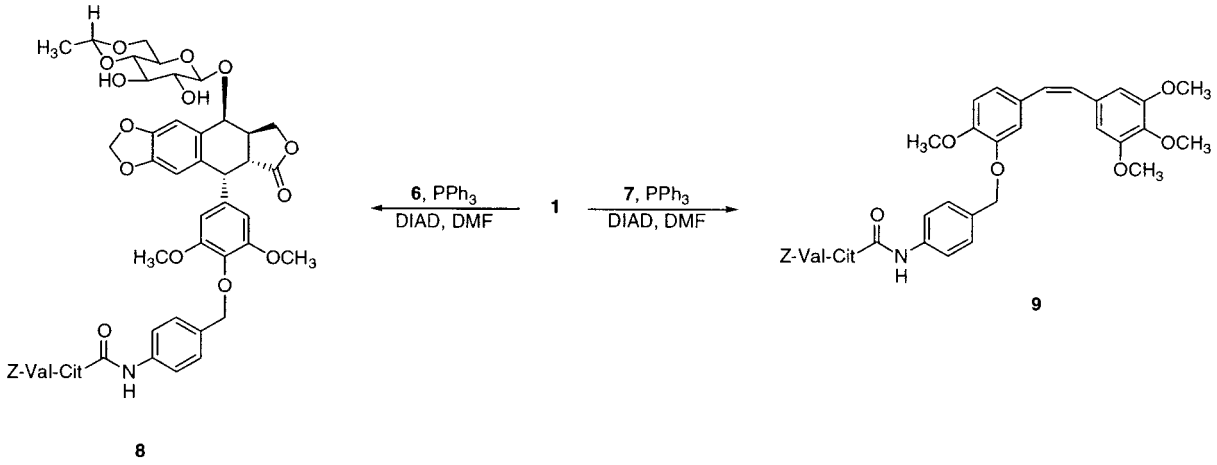
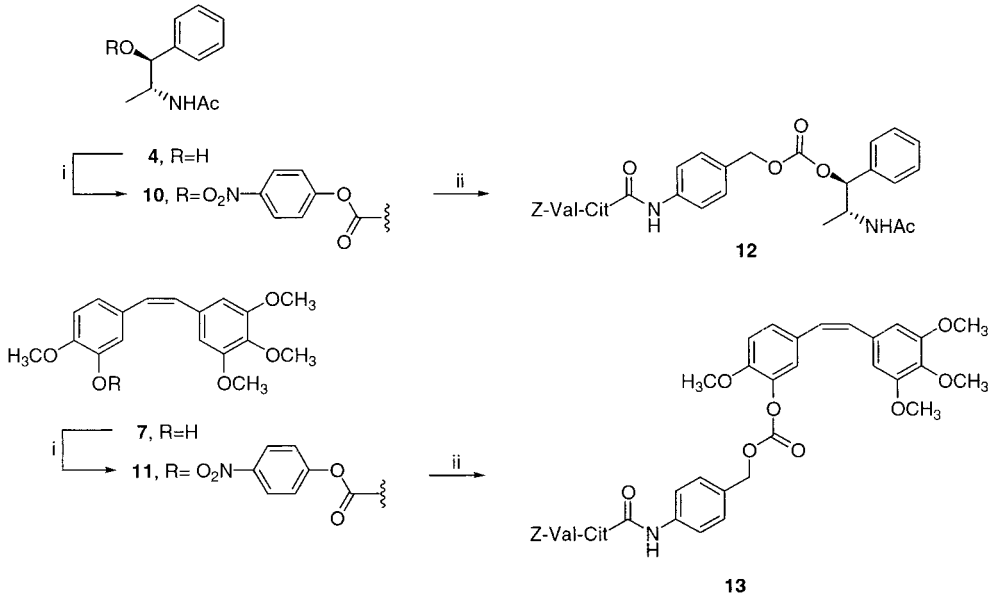
Scheme 4



Scheme 5



i. *p*-nitrophenylchloroformate, pyridine, DCM. ii. **1**, DMAP, DCM

combretastatin A-4 carbonate derivative **13** was as cytotoxic as combretastatin A-4 (**7**), reflecting the inherent instability of carbonate compared to that of the ether linkages (Table 1). These results, taken together with the enzyme hydrolysis studies, indicate that the peptide ether drug derivatives are prodrugs that can be activated by cathepsin B.

Discussion

Self-immolative linkers based on the *p*-aminobenzyl group have shown considerable utility for the attachment of amine-containing anticancer drugs to carriers that are cleaved by enzymes within tumors.[6−15,23] Variations on this methodology include the activation of *p*-nitrobenzyl carbamates upon biological reduction[29,30] and the 1,6-elimination reaction of *p*-mercaptobenzyl carbamates formed as a result of disulfide bond reduction.[31,32] The purpose of the work described here was to extend this linker methodology to include hydroxyl-based drug attachment, with the goal of producing highly stable anticancer prodrugs that can be activated by tumor-associated proteases. To our knowledge, the finding that combretastatin A-4 and etoposide *p*-aminobenzyl ethers undergo facile drug release constitutes the first demonstration that a 1,6-elimination reaction can effect the fragmentation of an ether bond. Furthermore, the application of this reaction pathway for the release of an anticancer drug from a stable peptide prodrug is new.

The advantage of using ethers rather than carbonates for drug attachment to peptides is evident from the stability results obtained with the combretastatin A-4 derivatives, **9** and **13**. While enzyme-catalyzed hydrolysis of the two compounds proceeded at similar rates, the carbonate **13** proved to be hydrolytically unstable and consequently was significantly more cytotoxic than **9**. It

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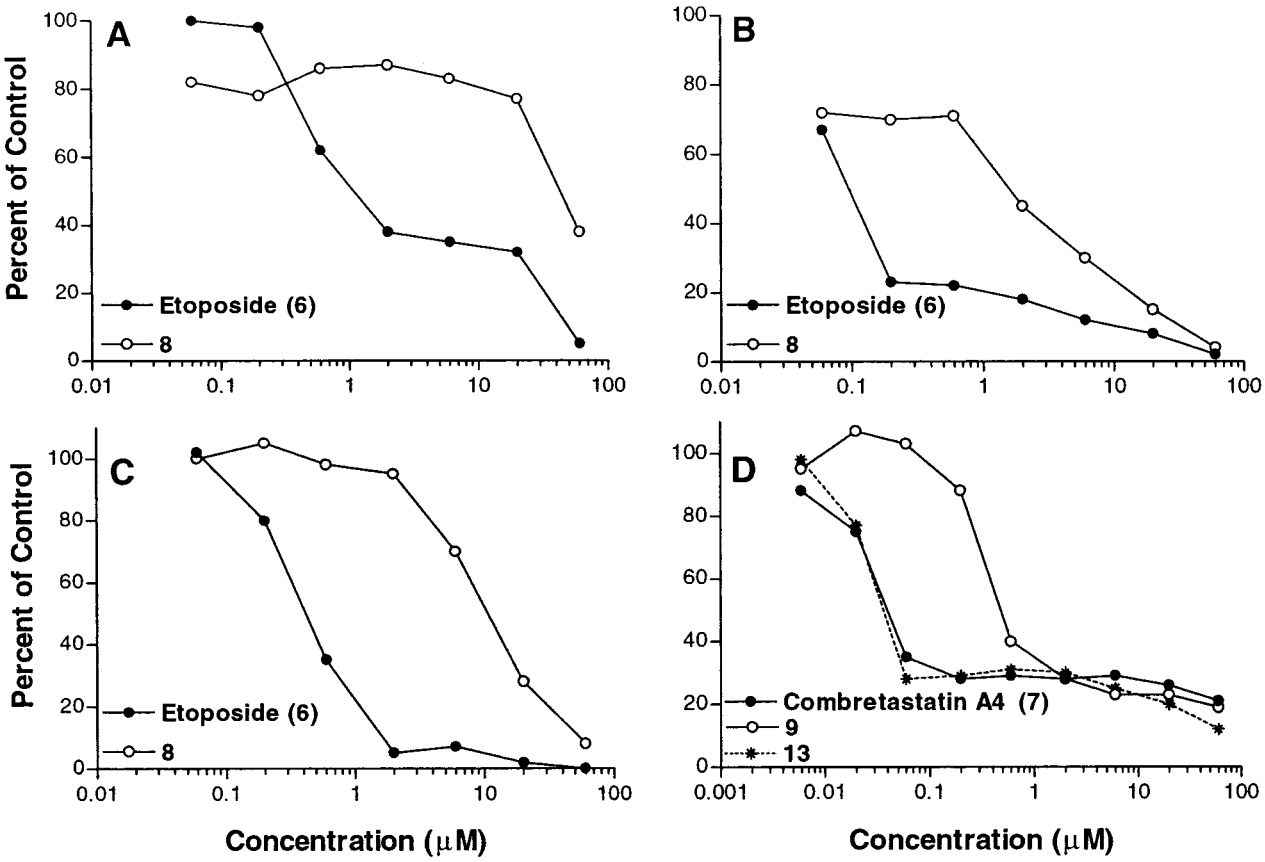

Figure 1. Cytotoxic effects on L2987 human lung adenocarcinoma (A and D), WM266/4 (B), and IGR-39 (C) human melanoma cell lines. The cells were exposed to various concentrations of the drugs for 24 h, washed, and incubated for another 48 h, and the cytotoxic activities were quantified through the incorporation of ^{3}H-thymidine relative to that in untreated control cells.

was expected that unstable carbonates[33] such as **13** would display nonspecific toxicities in vivo and would have limited application for selective activation by endogenous tumor proteases. On the other hand, the peptide ether derivatives **2**, **5**, **8**, and **9** showed no evidence of drug fragmentation after at least 1 week of incubation in human serum or in aqueous buffers at 37 °C. Treatment of **2**, **8**, and **9** with cathepsin B, an enzyme that is associated with growing and metastatic tumors,[1−3] led to the release of 1-naphthol or active anticancer drugs, with a concomitant increase in cytotoxic activity, which validates the application of protease-sensitive *p*-amidobenzyl ethers for the development of anticancer prodrugs. The failure of *N*-acetylnorephedrine (**5**) to undergo 1,6-elimination may be attributed to the high pK_a of the aliphatic alcohol-containing leaving group.

Further studies are necessary to explore the scope and the biological applications of the enzymatically induced reaction that leads to ether fragmentation. We demonstrated the feasibility of the reaction using cathepsin B, a lysosomal enzyme that is highly up-regulated in the cytosol and on the membranes of several metastatic cancers.[1−3] This enzyme is known to cleave the serum-stable val-cit sequence used in these studies but cleaves other peptide sequences such as val-lys and phe-lys much more rapidly.[10] Interestingly, the latter sequence is a substrate not only for cathepsin B but also for plasmin,[8−14] another lysosomal enzyme associated with various malignancies. We are investigating several such peptides, with the goal of finding particular sequences that lead to efficient intratumoral drug release and minimal release in nontarget tissues. In addition, investigation of other phenolic cytotoxic agents, such as the camptothecin family member SN-38,[34] phenol mustard,[35] minor groove binders related in structure to CC-1065,[36] streptonigrin,[37] and elliptininium acetate,[38] will expand the methodology to include drugs with widely different mechanisms of activities and structures. Some of these agents are among the most potent small molecular weight drugs known, and their corresponding prodrugs may be therapeutically effective in tumor types that are insensitive for kinetic reasons to prodrugs of etoposide and combretastatin A-4. In summary, we have demonstrated that *p*-aminobenzyl ethers undergo a facile 1,6-elimination reaction and that conditionally stable anticancer prodrugs can be developed on the basis of this reaction.

Experimental Section

General Methods. Commercially available reagents and solvents were obtained as follows: HPLC-grade solvents, Fisher; anhydrous solvents, Aldrich; diisopropyl azodicarboxylate (DIAD, 95%), Lancaster; 4-aminobenzyl alcohol, Alfa

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Aesar; Z-val-OSu, Advanced ChemTech; L-citrulline, Novabiochem; (*1S,2R*)-(+)-norephedrine and other commercially available reagents, Aldrich. Z-val-cit-PAB-OH (**1**)[10,11] and combretastatin A-4 (**7**)[39] were synthesized as previously described. ^{1}H NMR spectra were recorded on a Varian Gemini spectrophotometer at 300 MHz. Flash column chromatography was performed using 230−400 mesh ASTM silica gel from EM Science. Analtech silica gel GHLF plates were used for thin-layer chromatography. HPLC was performed using a Waters Alliance system with a photodiode array detector. Combustion analyses were determined by Quantitative Technologies, Inc., Whitehouse, NJ.

General Procedure for the Mitsunobu Reaction. Peptide **1** (1.0 equiv), triphenylphosphine (1.1 equiv) and the appropriate phenol (1.0−1.1 equiv) were dissolved in DMF/toluene (1:1) and evaporated to dryness under high vacuum. The residue was taken up in dry DMF while under N_2 and cooled to 0 °C. DIAD (1.1 equiv) was added dropwise over 1 min while stirring. The yellow-brown solution was monitored by TLC (9:1 CH_2Cl_2−MeOH). An additional 1.1 equiv of PPh_3 and DIAD was added after 4 h. The solution was stirred for a total of 16−24 h, followed by solvent removal in vacuo. The resulting product was purified by chromatography on silica gel (eluent gradient: 100% CH_2Cl_2 to 9:1 CH_2Cl_2−MeOH). The desired fractions were pooled and concentrated to a white or an off-white solid. Further purification could be obtained by triturating with ether.

Z-Val-cit-PAB-1-*O*-naphthol (2): R_f 0.26 (9:1 CH_2Cl_2−MeOH); mp 175 dec; UV λ_{max} 215, 242, 305 nm; LRMS (ESI$^+$) *m/z* 640.3 (M + H)$^+$, 662.2 (M + Na)$^+$, 678.2 (M + K)$^+$; ^{1}H NMR (DMSO-d_6) δ 8.19−8.24 (2 H, m, aromatic), 7.80−7.87 (2 H, m, aromatic), 7.06−7.50 (13 H, m, aromatic), 6.76 (1 H, d, J = 7.8 Hz, aromatic), 5.07 (2 H, s, Z-CH_2), 4.44−4.50 (1 H, m, val-CH), 4.28 (2 H, s, PAB-CH_2), 3.95 (1 H, d, J = 6.9 Hz, cit-CH), 3.03−3.20 (2 H, m, cit-NCH_2), 1.95−2.10 (1 H, m, val-CH), 1.28−1.78 (4 H, m, cit-CH_2's), 0.96 (3 H, d, J = 6.9 Hz, val-CH_3), 0.93 (3 H, d, J = 6.9 Hz, val-CH_3). Anal. ($C_{36}H_{41}N_5O_6$·H_2O) C, H, N.

Z-Val-cit-PAB-*O*-trichloroacetamidate (3). Peptide **1** (100 mg, 0.19 mmol) was dissolved in anhydrous DMF, and cesium carbonate (13 mg, 4 μmol, 0.2 equiv) was added. While under N_2, trichloroacetonitrile (0.2 mL, 1.9 mmol, 10 equiv) was added, and the contents were stirred while being monitored by TLC (9:1 CH_2Cl_2−MeOH) . The reaction was complete after 16 h. The mixture was filtered, and the filtrate was concentrated and subjected to chromatography on SiO_2 (eluent gradient 100% CH_2Cl_2 to 9:1 CH_2Cl_2−MeOH containing 1% triethylamine). The desired fractions were pooled and evaporated to an off-white powder (99 mg, 77%): R_f 0.44 (9:1 CH_2Cl_2−MeOH); UV λ_{max} 215, 250 nm; LRMS (ESI$^+$) *m/z* 679.3 (M + Na)$^+$, 681.2 (M + 2 + Na)$^+$, 683.2 (M + 4 + Na)$^+$, 685.2 (M + 6 + Na)$^+$, 695.2 (M + K)$^+$, 697.2 (M + 2 + K)$^+$, 699.2 (M + 4 + K)$^+$; ^{1}H NMR (DMSO-d_6) δ 10.08 (1 H, s, PAB-NH), 9.37 (1 H, s, C=NH), 8.10 (1 H, d, J = 7.8 Hz, amide NH), 7.60 (2 H, d, J = 8.4 Hz, PAB-CH ×2), 7.21−7.38 (8 H, m, aromatic), 5.96 (1 H, t, J = 5.1 Hz, cit-NH), 5.40 (2 H, s, cit-NH_2), 5.21 (2 H, s, PAB-CH_2), 5.02 (2 H, s, Z-CH_2), 4.40 (1 H, dd, J = 13.2, 7.8 Hz, val-CH), 3.90 (1 H, t, J = 8.4 Hz, cit-CH), 2.85−3.15 (2 H, m, cit-CH_2), 1.90−2.05 (1 H, m, val-CH), 1.28−1.74 (4 H, m, cit-CH_2), 0.86 (3 H, d, J = 6.6 Hz, val-CH_3), 0.82 (3 H, d, J = 6.9 Hz, val-CH_3). Anal. ($C_{28}H_{35}Cl_3N_6O_6$·0.2H_2O, 0.4Et_3N) C, H, N, Cl.

***(1S,2R)*-*N*-Acetyl-norephedrine (4).** (*1S,2R*)-(+)-norephedrine (5.0 g, 32.4 mmol) was partially suspended in water (65 mL, 0.5 M). Acetic anhydride (6.2 mL, 64.8 mmol, 2.0 equiv) was added, and the resulting yellow solution was stirred for 1 h. EtOAc was added, the layers were separated, and the aqueous layer was further washed with EtOAc (2×). The combined organic extracts were washed with brine and dried ($MgSO_4$). Filtration, followed by removal of solvent, led to a yellow oil that slowly formed yellow crystals. The crude product was purified by chromatography on SiO_2 (1:1 CH_2Cl_2−EtOAc), and the combined fractions were concentrated to a clear oil that solidified. Recrystallization from EtOAc−hexanes gave a white cottonlike solid as the desired product (4.95 g, 79%): mp 123 °C; R_f 0.14 (1:1 CH_2Cl_2−EtOAc); UV λ_{max} 215, 256 nm; ^{1}H NMR ($CDCl_3$) δ 7.28−7.39 (5 H, m, aromatic), 5.59 (1 H, br d, J = 8.4 Hz, NH), 4.87 (1 H, d, J = 3.6 Hz, H-1), 4.34 (1 H, dp, J = 3.0, 6.9 Hz, H-2), 3.48 (1 H, br s, OH), 2.01 (3 H, s, Ac), 1.02 (3 H, d, J = 6.9 Hz, H-3). Anal. ($C_{11}H_{15}NO_2$) C, H, N.

Z-Val-cit-PAB-*O*-(*N*-Ac)-Nor (5). The trichloroacetamidate **3** (1 equiv) and alcohol **4** (1 equiv) were suspended in anhydrous CH_2Cl_2 and cooled to 0 °C. Dropwise addition of trifluoromethanesulfonic acid (0.5 equiv) gave an immediate gummy precipitate. TLC analysis (9:1 CH_2Cl_2−MeOH) showed a product (R_f 0.28) and some decomposition of **3** to Z-val-cit-PAB-OH **1** (R_f 0.14). The contents were evaporated to a yellow solid and purified by chromatography on SiO_2 (eluent gradient 100% CH_2Cl_2 to 9:1 CH_2Cl_2−MeOH). The desired ether (**5**) was isolated as an off-white solid after triturating with diethyl ether: R_f 0.28 (9:1 CH_2Cl_2−MeOH); UV λ_{max} 215, 256 nm; LRMS (ESI$^+$) *m/z* 688.4 (M + H)$^+$, 711.4 (M + Na)$^+$; ^{1}H NMR (DMSO-d_6) δ 10.02 (1 H, s, PAB-NH), 8.10 (1 H, d, J = 7.2 Hz, amide NH), 7.81 (1 H, d, J = 9.0 Hz, amide NH), 7.56 (2 H, d, J = 8.7 Hz, PAB-CH ×2), 7.21−7.39 (12 H, m, aromatic), 5.98 (1 H, t, J = 5.1 Hz, cit-NH), 5.41 (2 H, s, cit-NH_2), 5.03 (2 H, s, Z-CH_2), 4.06−4.45 (4 H, m, val-CH, Nor-CH, PAB-CH_2), 3.84−3.94 (2 H, m, cit-CH, Nor-CH), 2.85−3.15 (2 H, m, cit-CH_2), 1.87−2.04 (1 H, m, val-CH), 1.67 (3 H, s, Nor-Ac), 1.28−1.75 (4 H, m, cit-CH_2's), 0.98 (3 H, d, J = 6.6 Hz, Nor-CH_3), 0.86 (3 H, d, J = 6.6 Hz, val-CH_3), 0.82 (3 H, d, J = 6.9 Hz, val-CH_3). Anal. ($C_{37}H_{48}N_6O_7$·H_2O) C, H, N.

Z-Val-cit-PAB-*O*-etoposide (6). Following the Mitsunobu procedure described above, the pure fractions from chromatography on SiO_2 gave the ether as a white solid (64%): R_f 0.29 (9:1 CH_2Cl_2−MeOH); UV λ_{max} 215, 250, 290 nm; LRMS (ESI$^+$) *m/z* 1084.6 (M + H)$^+$, 1106.6 (M + Na)$^+$, 1122.6 (M + K)$^+$; ^{1}H NMR (DMSO-d_6) δ 10.01 (1 H, s, PAB-NH), 8.08 (1 H, d, J = 7.2 Hz, amide NH), 7.57 (2 H, d, J = 8.1 Hz, PAB-CH ×2), 7.29−7.40 (7 H, m, aromatic), 7.00 (2 H, s, etop aromatic), 6.53 (1 H, s, etop aromatic), 6.23 (2 H, s, etop aromatic), 6.01 (1 H, d, J = 3.3 Hz, etop-CH_2), 5.96 (1 H, t, J = 5.1 Hz, cit-NH), 5.40 (2 H, s, cit-NH_2), 5.24 (1 H, s, etop-OH), 5.22 (1 H, s, etop-OH), 5.02 (2 H, s, Z-CH_2), 4.92 (1 H, d, J = 3.0 Hz, etop-CH), 4.74 (2 H, s, PAB-CH_2), 4.70 (1 H, dd, J = 9.9, 4.8 Hz, etop-CH), 4.56 (1 H, d, J = 7.8 Hz, etop-CH), 4.54 (1 H, d, J = 5.1 Hz, etop-CH), 4.36−4.44 (1 H, m, val-CH), 4.25 (2 H, dd, J = 9.0 Hz, etop-CH ×2), 4.06 (1 H, dd, J = 11.1, 4.8 Hz, etop-CH), 3.90 (1 H, t, J = 6.9 Hz, cit-CH), 3.62 (6 H, s, etop-OCH_3 ×2), 3.49 (1 H, t, J = 9.6 Hz, etop-CH), 2.81−3.30 (9 H, m, etop-CH ×7, cit-NCH_2), 1.88−2.05 (1 H, m, val-CH), 1.30−1.74 (4 H, m, cit-CH_2's), 1.22 (3 H, d, J = 4.8 Hz, etop-CH_3), 0.86 (3 H, d, J = 6.6 Hz, val-CH_3), 0.82 (3 H, d, J = 6.9 Hz, val-CH_3). Anal. ($C_{55}H_{65}N_5O_{18}$·2H_2O) C, H, N.

Z-Val-cit-PAB-3′-*O*-combretastatin A-4 (9). Using the Mitsunobu reaction conditions described above, the compound was isolated as an amorphous solid after trituration. R_f 0.42 (9:1 CH_2Cl_2−MeOH); mp 169−172 dec; UV λ_{max} 215, 248, 300 nm; LRMS (ESI$^+$) *m/z* 812.4 (M + H)$^+$, 834.4 (M + Na)$^+$, 850.4 (M + K)$^+$; ^{1}H NMR (DMSO-d_6) δ 10.06 (1 H, s, PAB-NH), 8.11 (1 H, d, J = 7.2 Hz, amide NH), 7.57 (2 H, d, J = 8.4 Hz, PAB-CH ×2), 7.24−7.45 (6 H, m, aromatic), 7.21 (2 H, d, J = 8.4 Hz, PAB-CH ×2), 6.82−6.98 (2 H, m, CSA4-H-5′, -6′), 6.56 (2 H, s, CSA4-H-2), 6.48 (2 H, d, J = 12.3 Hz, CSA4-*cis*-CH), 6.44 (2 H, d, J = 12.3 Hz, CSA4-*cis*-CH), 5.97 (1 H, t, J = 5.1 Hz, cit-NH), 5.41 (2 H, s, cit-NH_2), 5.03 (2 H, s, Z-CH_2), 4.76 (2 H, s, PAB-CH_2), 4.36−4.45 (1 H, m, val-CH), 3.92 (1 H, t, J = 7.2 Hz, cit-CH), 3.82 (3 H, s, CSA4-3′-OCH_3), 3.61 (9 H, s, CSA4-3,4,5-OCH_3), 2.88−3.07 (2 H, m, cit-NCH_2), 1.90−2.03 (1 H, m, val-CH), 1.28−1.78 (4 H, m, cit-CH_2's), 0.86 (3 H, d, J = 6.6 Hz, val-CH_3), 0.82 (3 H, d, J = 6.9 Hz, val-CH_3). Anal. ($C_{44}H_{53}N_5O_{10}$·H_2O) C, H, N.

***(1S,2R)*-*N*-Acetyl-*O*-(4-nitrophenyloxycarbonyl)norephedrine (10).** Compound **4** (1.0 g, 5.17 mmol, 1.0 equiv) and *p*-nitrophenylchloroformate (1.61 g, 7.76 mmol, 1.5 equiv) were dissolved in anhydrous THF (12 mL, 0.5 M) while under

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N_2. Dry pyridine (0.63 mL, 7.76 mmol, 1.0 equiv) was added via syringe over a 3 min period. The resulting turbid mixture contained no starting material after 15 min, according to TLC (1:1 CH_2Cl_2−EtOAc). Solids were filtered off and washed with THF. The filtrate was concentrated to a yellow oil that was purified by chromatography on SiO_2 (1:1 hexanes−EtOAc). The desired product **10** was an off-white solid (1.43 g, 78%) that was stored in the dark at <0 °C: R_f 0.16 (1:1 hexanes−EtOAc); UV λ_{max} 215, 270 nm; ^{1}H NMR ($CDCl_3$) δ 8.24 (2 H, d, J = 9.3 Hz, Pnp-CH ×2), 7.38 (2 H, d, J = 9.0 Hz, Pnp-CH ×2), 7.32−7.44 (5 H, m, aromatic), 5.78 (1 H, d, J = 3.3 Hz, H-1), 5.42 (1 H, br d, J = 8.4 Hz, NH), 4.61 (1 H, dp, J = 3.3, 7.2 Hz, H-2), 2.00 (3 H, s, Ac), 1.11 (3 H, d, J = 7.2 Hz, H-3).

3′-*O*-(4-Nitrophenyloxycarbonyl)combretastatin A-4 (11). Using the same procedure as described above, combretastatin A-4 (120 mg, 0.38 mmol) was converted to the 4-nitrophenyl carbonate in quantitative yield (183 mg) and isolated as a yellow oil: R_f 0.47 (3:2 hexanes−EtOAc); ^{1}H NMR ($CHCl_3$) δ 8.30 (2 H, d, J = 9.3 Hz, Pnp-CH ×2), 7.45 (2 H, d, J = 9.3 Hz, Pnp-CH ×2), 7.18 (2 H, d, J = 1.5 Hz, H-2′), 6.88−6.94 (2 H, m, H-5′,-6′), 6.51 (1 H, d, J = 12.0 Hz, *cis*-CH), 6.49 (2 H, s, H-2), 6.48 (1 H, d, J = 12.0 Hz, *cis*-CH), 3.89 (3 H, s, 3′-OCH_3), 3.84 (3 H, s, 4-OCH_3), 3.70 (3 H, s, 3,5-OCH_3).

Z-Val-cit-PAB-OCO-(*1S,2R*)-(*N*-acetyl)norephedrine (12). The activated carbonate **10** (90 mg, 0.25 mmol) and Z-val-cit-PAB-OH **1** (130 mg, 0.25 mmol) were suspended in dry CH_2Cl_2 (8 mL), followed by the addition of DMAP (34 mg, 0.28 mmol, 1.1 equiv). The reaction was stopped after 26 h by the addition of EtOAc and 10% citric acid. The layers were separated, and the organic phase was further washed with water and brine. A precipitate formed that was filtered and added to the separated EtOAc layer and concentrated. The resulting yellow solid was subjected to chromatography on SiO_2 (gradient eluent 95:5 to 9:1 CH_2Cl_2−MeOH). The desired product eluted first and was concentrated to a white flaky solid (35 mg, 19%), while Z-val-cit-PAB-OH (**1**) was recovered as the second eluate: R_f 0.17 (9:1 CH_2Cl_2−MeOH); UV λ_{max} 215, 256 nm; LRMS (ESI$^+$) m/z 792.5 $(M + H)^+$, 814.5 $(M + Na)^+$, 830.4 $(M + K)^+$; ^{1}H NMR (DMSO-d_6) δ 10.08 (1 H, s, PAB-NH), 8.10 (1 H, d, J = 7.2 Hz, amide NH), 8.00 (1 H, d, J = 7.8 Hz, amide NH), 7.59 (2 H, d, J = 8.7 Hz, PAB-CH ×2), 7.26−7.39 (10 H, m, aromatic), 7.24 (2 H, d, J = 8.7 Hz, PAB-CH ×2), 5.96 (1 H, t, J = 5.1 Hz, cit-NH), 5.61 (1 H, d, J = 4.2 Hz, Nor-CH), 5.40 (2 H, s, cit-NH_2), 5.07 (2 H, s, Z-CH_2), 5.03 (2 H, s, PAB-CH_2), 4.40 (1 H, dd, J = 13.2, 7.8 Hz, val-CH), 4.03−4.14 (1 H, m, Nor-CH), 3.92 (1 H, t, J = 7.8 Hz, cit-CH), 2.85−3.06 (2 H, m, cit-CH_2), 1.90−2.02 (1 H, m, val-CH), 1.74 (3 H, s, Nor-Ac), 1.28−1.75 (4 H, m, cit-CH_2's), 0.96 (3 H, d, J = 6.9 Hz, Nor-CH_3), 0.87 (3 H, d, J = 6.9 Hz, val-CH_3), 0.83 (3 H, d, J = 7.2 Hz, val-CH_3). Anal. ($C_{38}H_{48}N_6O_9 \cdot {}^1/_2H_2O$) C, H, N.

Z-Val-cit-PAB-OCO-combretastatin A-4 (13). Activated combretastatin A-4 **11** (120 mg, 0.25 mmol) and Z-val-cit-PAB-OH **1** (130 mg, 0.25 mmol) were suspended in dry CH_2Cl_2/pyridine (3 mL each), followed by the addition of DMAP (34 mg, 0.28 mmol, 1.1 equiv). The reaction was sonicated for 2 h, followed by stirring for 20 h. Evaporation of the reaction mixture, followed by purification by chromatography on SiO_2 (gradient eluent 100% CH_2Cl_2 to 9:1 CH_2Cl_2−MeOH) and concentration of the appropriate fractions, resulted in a yellow oil that was precipitated from CH_2Cl_2 (1 mL) through the addition of ether, which led to a yellow solid (83 mg, 38%): R_f 0.47 (9:1 CH_2Cl_2−MeOH); mp 155−158 dec; UV λ_{max} 215, 245, 285 nm; LRMS (ESI$^+$) m/z 856.5 $(M + H)^+$, 878.5 $(M + Na)^+$, 894.5 $(M + K)^+$; ^{1}H NMR (DMSO-d_6) δ 10.10 (1 H, s, PAB-NH), 8.11 (1 H, d, J = 7.8 Hz, amide NH), 7.62 (2 H, d, J = 8.1 Hz, PAB-CH ×2), 7.25−7.40 (8 H, m, aromatic), 7.15 (1 H, dd, J = 8.7, 1.8 Hz, CSA4-H-6′), 7.07 (1 H, d, J = 8.1 Hz, CSA4-H-5′), 7.06 (1 H, d, J = 2.4 Hz, CSA4-H-2′), 6.51 (2 H, s, CSA4-H-2), 6.48 (2 H, s, CSA4-CH=CH), 5.97 (1 H, t, J = 5.1 Hz, cit-NH), 5.42 (2 H, s, cit-NH_2), 5.14 (2 H, s, Z-CH_2), 5.02 (2 H, s, PAB-CH_2), 4.40 (1 H, dd, J = 12.9, 8.1 Hz, val-CH), 3.92 (1 H, t, J = 7.2 Hz, cit-CH), 3.73 (3 H, s, CSA4-3′-OCH_3), 3.61 (3 H, s, CSA4-4-OCH_3), 3.58 (6 H, s, CSA4-3,5-OCH_3), 2.88−3.07 (2 H, m, cit-NCH_2), 1.88−2.04 (1 H, m, val-CH), 1.28−1.178 (4 H, m, cit-CH_2's), 0.86 (3 H, d, J = 6.9 Hz, val-CH_3), 0.82 (3 H, d, J = 6.6 Hz, val-CH_3). Anal. ($C_{45}H_{53}N_5O_{12} \cdot H_2O$) C, H, N.

General Procedure for Cathepsin B Assays. Bovine spleen cathepsin B was obtained from Sigma-Aldrich. SDS-PAGE analysis revealed bands corresponding to molecular masses of 24 and 29 kDa, as expected for the fully processed protein.[40] Enzyme activity was assayed using the cathepsin B substrate Z-lysine nitrophenyl ester, according to a published procedure.[41] The average specific activity was 26 μmol/min/mg of enzyme, which fell within the indicated range of 18−35 μmol/min/mg. The enzyme, dissolved in phosphate-buffered saline (pH 7.2, 1 mg/mL final concentration), was activated with dithiothreitol and EDTA, as previously described.[41] A 1.0 mM stock solution of the peptide substrate in DMSO was added to acetate buffer (25 mM) containing 1 mM EDTA (pH 5.1) to a final concentration of 0.08−0.14 mM, and to this solution was added the activated enzyme (12−15 μg/mL). In the case of the naphthol ether **2**, a 5.0 mM solution in MeOH was diluted to a final concentration of 0.22 mM. Periodically, aliquots were taken, quenched with an equal volume of MeCN, and centrifuged, and 100 μL injections were analyzed by HPLC (4.6 mm × 15 cm C_{18} column) with detection between 210 and 400 nm. The mobile phase consisted of (A) 5 mM sodium phosphate (pH 7) and (B) either MeOH (for compounds **2**, **9**, and **13**) or MeCN (for compounds **5**, **8**, and **12**). The gradient elution was 90% to 10% A over 10 min, followed by 5 min at 10% A, and the flow rate was 1.0 mL/min. The disappearance of substrate and the appearances of released alcohol and Z-val-cit were recorded. Cathepsin B hydrolysis rates were calculated according to the disappearance of substrate (Table 1).

General Procedure for All Stability Studies. Solutions of the substrates (0.08−0.14 mM in DMSO, and 0.22 mM in MeOH for **2**) were diluted 10−20-fold in PBS, acetate buffer (25 mM, pH 5.1), or pooled human serum, and incubation was carried out at 37 °C. For the serum studies, equal volumes of MeCN were added, and the samples were centrifuged prior to HPLC analysis. The other samples were injected directly into the HPLC.

In Vitro Cytotoxicity Assays. L2987 human lung adenocarcinoma cells were obtained as previously described.[42] WM266/4 and IGR-39 human melanoma cells were obtained from ATCC (Manassas, VA) and DSMZ (Braunschweig, Germany), respectively. L2987 and WM266/4 cells were grown in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, 10 units/mL penicillin G, and 10 μg/mL streptomycin sulfate. Dulbecco's modified Eagle's medium was used in place of RPMI for the IGR-39 cells. The cells (2500 cells in 0.1 mL of medium) were plated into 96-well plates, and after 24 h at 37 °C, various concentrations of the drugs in the medium (50 μL) were added in triplicate. Incubation was continued for an additional 24 h, the cultures were washed, and fresh medium (0.15 mL) was added. After 48 h at 37 °C, ^{3}H-thymidine (25 μL, 0.5 μCi/well) was added, and the cultures were frozen, thawed, and harvested onto glass fiber filters 4 h later. Incorporation of the label into nascent DNA was measured using a β-counter.

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