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Peptidyl α-Ketoamides with Nucleobases, Methylpiperazine, and Dimethylaminoalkyl Substituents as Calpain Inhibitors

Asli Ovat,[†,‡] Zhao Zhao Li,[†,‡] Christina Y. Hampton,[†] Seneshaw A. Asress,[§] Facundo M. Fernández,[†] Jonathan D. Glass,[§,∥] and James C. Powers*[,†,‡]

[†]*School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, Georgia 30332,* [‡]*Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, Georgia 30332,* [§]*Center for Neurodegenerative Disease, Emory University School of Medicine, 615 Michael Street, Atlanta, Georgia 30322, and* [∥]*Department of Neurology, Emory University School of Medicine, 615 Michael Street, Atlanta, Georgia 30322*

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A series of peptidyl α-ketoamides with the general structure Cbz-L-Leu-D,L-AA-CONH-R were synthesized and evaluated as inhibitors for the cysteine proteases calpain I, calpain II, and cathepsin B. Nucleobases, methylpiperazine, and dimethylaminoalkyl groups were incorporated into the primed region of the inhibitors to generate compounds that potentially cross the blood−brain barrier. Two of these compounds (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl and Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) have been shown to have useful concentrations in the brain in animals. The best inhibitor for calpain I was Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (K_i = 23 nM), and the best inhibitor for calpain II was Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl (K_i = 68 nM). On the basis of the crystal structure obtained with heterocyclic peptidyl α-ketoamides, we have improved inhibitor potency by introducing a small hydrophobic group on the adenine ring. These inhibitors have good potential to be used in the treatment of neurodegenerative diseases.

Introduction

Calpains are cysteine proteases that require calcium for activation. They belong to Clan CA of cysteine proteases together with cruzain, rhodesain, papain, and cathepsins. There are multiple isoforms of calpain that are both ubiquitous and tissue specific. Calpain I (μ-calpain) and calpain II (m-calpain) are the two major calpain isoforms that are widely distributed in mammalian cells. These two isoforms are very similar and differ in the calcium concentration that they require to become activated. Calpain I is activated by micromolar concentrations of Ca^{2+}, whereas calpain II is activated by millimolar concentrations of Ca^{2+}. Calpains are involved in a variety of calcium-regulated biological processes, such as cell proliferation and differentiation, apoptosis, membrane fusion, signal transduction, and platelet activation. Enhanced calpain activity has been observed in a number of diseases including ischemic[1,2] and traumatic[3,4] brain injury, cancer,[5−7] muscular dystrophy,[8,9] cataracts,[10] strokes,[11] and neurological disorders like Alzheimer's,[12,13] Huntington's,[14] and Parkinson's[15,16] diseases and multiple sclerosis.[17,18] Involvement of calpains in a wide variety of biological processes and diseases makes them important targets for the development of inhibitors. There are several reviews on the roles of calpains in diseases.[19−26]

Synthetic calpain inhibitors can be divided into two groups: peptidic inhibitors and nonpeptidic inhibitors. Peptidic inhibitors can be further divided into two groups: reversible inhibitors and irreversible inhibitors. Peptidyl aldehydes,[27−35] α-keto-acids,[36,37] α-ketoesters,[36] α-ketoamides,[36,38−40] α-diketones,[41] and α-keto phosphorus[42] are examples of reversible peptidyl inhibitors, whereas peptidyl epoxysuccinates,[43−45] vinyl sulfones,[46] acyloxymethyl ketones,[47] diazomethyl ketones,[48] and chloromethyl ketones[49] are examples of irreversible peptidyl inhibitors of calpain. Reversible inhibitors of calpain are favored over the irreversible inhibitors for drug development, since there are many isoforms of calpains and nonspecific inhibition of these isoforms can cause severe side effects. Calpain inhibitors have been reviewed.[50−52]

Synthetic calpain inhibitors protect against neuronal loss and improve neurological function in animal models of Alzheimer's disease,[53] traumatic brain injury,[54] chronic progressive experimental autoimmune encephalomyelitis,[55] cerebral ischemia,[56] optic nerve degeneration,[57] spinal cord injury,[58,59] and paclitaxel-induced sensory neuropathy.[60] In addition, calpain inhibitors are effective for the treatment of cataracts[61] and have antimalarial activity.[62] The neuroprotective effects of calpain inhibitors are well established, but their use in treatment of human diseases is challenged by their inability to cross the blood−brain barrier (BBB[a]).

*To whom correspondence should be addressed. Phone: (404) 894-4038. Fax: (404) 894-2295. Email: james.powers@chemistry.gatech.edu.

[a] Abbreviations: Abu, 4-aminobutyric acid; AMC, 7-amino-4-methylcoumarin; BBB, blood−brain barrier; Brij, polyoxyethylene lauryl ether; Bzl, benzyl, CH_2Ph; Cbz, benzyloxycarbonyl; $CDCl_3$, deuterated chloroform; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHT, high affinity choline transporter; CNS, central nervous system; CNT, concentrative nucleoside transporters; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DMSO-d_6, deuterated dimethyl sulfoxide; DTT, dithiothreitol; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Et_2O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, *N*-hydroxybenzotriazole; iBCF, isobutyl chloroformate; LC−MS, liquid chromatography−mass spectrometry; LC−MS/MS, liquid chromatography−tandem mass spectrometry; MeOH, methanol; Nva, norvaline; NMM, *N*-methylmorpholine; OBzl, benzyloxy; Phe, phenylalanine; RT, room temperature; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane; RFU, relative fluorescence unit; VAChT, vesicular acetylcholine transporter.

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The BBB is a structural and physiological barrier that restricts the passage of various chemical substances into the central nervous system (CNS).[63] The "protective" function of the BBB is also a major obstacle to the delivery of pharmacologic agents to the CNS for the treatment of neurological disorders. Our lead compound, Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-morpholine (AK295, **1**, Figure 1), is a reversible peptidyl α-ketoamide calpain inhibitor that is neuroprotective in models of head trauma,[54] focal brain ischemia,[64] and axonal degeneration caused by axotomy or exposure to vincristine[65] and paclitaxel.[60] The data document the potential for AK295 to be a potentially effective compound for the treatment of human disease, but the development of **1** as a drug may be hampered by its inability to cross the BBB. In order to design new analogues of **1** that may cross the BBB, we replaced the morpholine ring with structural features that could be recognized by the intrinsic BBB transport systems.

Here we describe new calpain inhibitors that contain nucleobases, methylpiperazine, and dimethylaminoalkyl moieties in the primed region of the inhibitor. We hypothesized that these compounds could be recognized by BBB transport systems in the brain and thus would penetrate into the brain and spinal cord to inhibit calpain activation during the progression of neurological diseases.

Chemistry

We previously reported synthetic methods for the preparation of peptidyl α-ketoamides.[36,38] The α-ketoesters Cbz-Leu-D,L-Abu-CO_2Et and Cbz-Leu-D,L-Phe-CO_2Et were prepared by a two-step Dakin−West reaction from the corresponding dipeptide acids Cbz-Leu-Abu-OH and Cbz-Leu-Phe-OH. The dipeptide acids were reacted with ethyloxalyl chloride in the presence of pyridine and 4-dimethylaminopyridine (DMAP) to form peptidyl α-enol esters. The peptidyl α-enol esters were then converted to peptidyl α-ketoesters by reacting with triethylamine. The peptidyl α-ketoacids were obtained by the hydrolysis of the peptidyl α-ketoesters with 1 M NaOH under standard deblocking conditions to give **2** and **3** (Figure 2).

Some of the P′ amines such as *N*,*N*-dimethylpropane-1,3-diamine and *N*,*N*-dimethylethane-1,2-diamine were commercially available. Other amines such as 9-(3-aminopropyl)-adenine[66] and 9-(3-aminopropyl)-2-methoxyadenine were synthesized in three steps using the procedure described by Woollins and co-workers.[66] Reaction of 2-chloroadenine (**6**) with sodium methoxide gave 2-methoxyadenine (**7**).[67] Adenine and 2-methoxyadenine (**7**) were reacted with 1-bromo-3-chloropropane to add the linker by a single alkylation reaction. The chloro group on the linker was then reacted with sodium azide to obtain the corresponding azide derivatives. Catalytic reduction of the azide in the presence of palladium activated on carbon and hydrogen gas gave the precursor amines 9-(3-aminopropyl)adenine and 9-(3-aminopropyl)-2-methoxyadenine (**8**). For the synthesis of 1-(3-aminopropyl)cytosine,[68] *N*-acetylcytosine was reacted with 1-bromo-3-chloropropane and then with sodium azide to form 1-(3-azidopropyl)-*N*-acetylcytosine. The acetyl group was deblocked with the ammonia, and then catalytic reduction of azide to the amine was completed in the presence of palladium activated on carbon and hydrogen gas. For the synthesis of 1-(3-aminopropyl)-4-methylpiperazine,[69] *N*-methylpiperazine was reacted with *N*-(3-bromopropyl)phthalimide and the corresponding amine was obtained after reacting *N*-(3-(4-methylpiperazin-1-yl)propyl)phthalimide with hydrazine monohydrate.

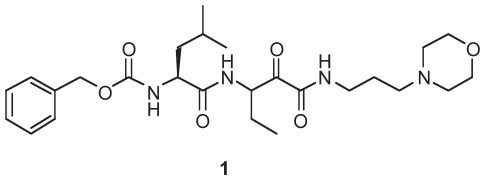

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Figure 1. Structure of Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-morpholinyl (**1**).

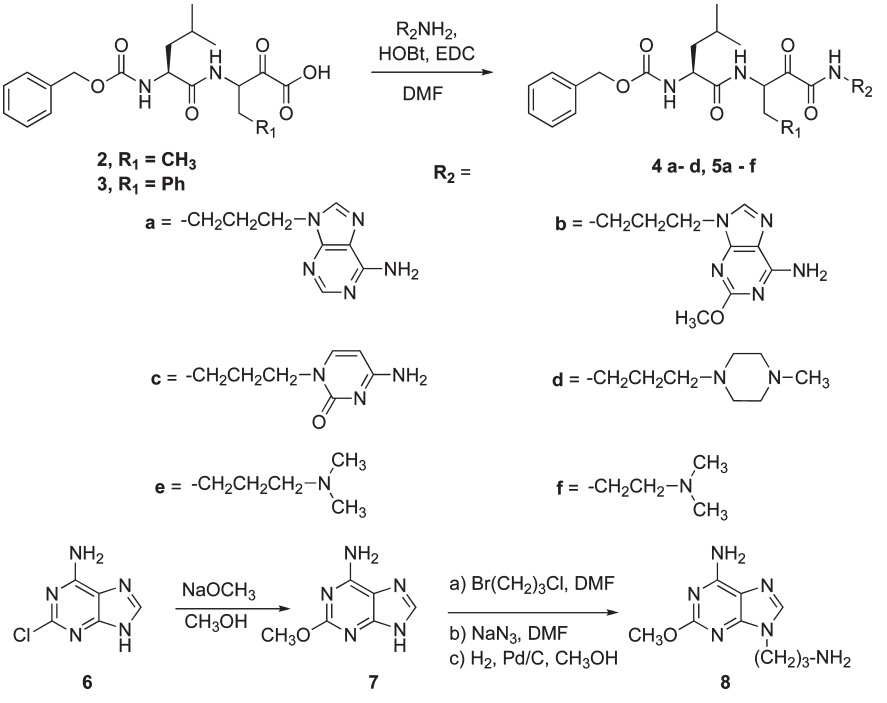

Figure 2. Synthesis of the peptidyl α-ketoamides.

The target peptidyl α-ketoamides were obtained in low yields by coupling the appropriate α-ketoacid (**2**, **3**) and the appropriate amine (R_2NH_2) using *N*-hydroxybenzotriazole (HOBt) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Figure 2).

Results and Discussion

Synthetic Design. We designed a series of new peptidyl α-ketoamides extending to the primed region as calpain inhibitors. Peptidyl α-ketoamides inactivate the cysteine proteases by forming a reversible hemithioketal adduct with the active site cysteine residue that resembles the transition state for peptide bond hydrolysis. This intermediate is quite stable, and thus, low K_i values have been observed with peptidyl α- ketoamide transition-state inhibitors. Peptidyl α-ketoamides have been extensively studied by our group[36,38] and other investigators.[70−73] Our previous work has shown that extension of the inhibitors to the primed region increased the potency of the inhibitors and N-monosubstituted peptidyl α-ketoamides were more potent than the corresponding N,N-disubstituted peptidyl α-ketoamides.[38] It has also been observed that α-aminobutyric acid (Abu), phenylalanine (Phe), or norvaline (Nva) in the P1 position and leucine in the P2 position were preferred.[74] To generate compounds capable of crossing the BBB, we designed peptidyl α-ketoamides with nucleobases, methylpiperazine, and dimethylaminoalkyl structures in the primed region.

Charged and polar compounds such as nucleotides and choline, which are essential for the brain, do not cross the BBB, and there are multiple transport systems to facilitate the delivery of these compounds to the brain.[75] Choline, a positively charged molecule, has a critical role in the CNS as a precursor to the neurotransmitter acetylcholine but does not cross the BBB, and its uptake into the brain is dependent upon carrier-mediated transport. Several BBB choline transporters such as the high affinity choline transporter (CHT) and the vesicular acetylcholine transporter (VAChT) are responsible for the transport of choline across the BBB.[76] The choline transporters also deliver choline analogues such as *N*-*n*-octylcholine, *N*-*n*-decylnicotinium iodide, bis-pyridinium cyclophanes,[77] and nicotine to the brain.[78] Several cationic drugs such as verapamil, diphenhydramine, and donepezil are transported by or are competitive inhibitors of the choline transporters.[79,80] We synthesized several peptidyl α-ketoamides Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) (**4d**), Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) (**5d**), Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$ (**5e**), and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$ (**5f**) that contain *N*-methylpiperazine or dimethylaminoalkyl groups in the primed region. The methylpiperazine derivatives are similar to our lead structure **1** where there is a P′ morpholine ring but not a methylated tertiary amine. The methylpiperazine ring has some features in common with donepezil (a nitrogen heterocycle), verapamil (a methyl tertiary amine), and nicotine (both). It was hypothesized that these structural features would provide sufficient recognition for some of the choline transporters to enable these compounds to penetrate the brain. The dimethylaminoalkyl moiety in ketoamides **5e** and **5f** is found in diphenhydramine and could also be recognized by the choline transporters. We planned to methylate these compounds to increase their resemblance to choline but abandoned that strategy when the dimethylaminoalkyl compounds proved to be difficult to purify and

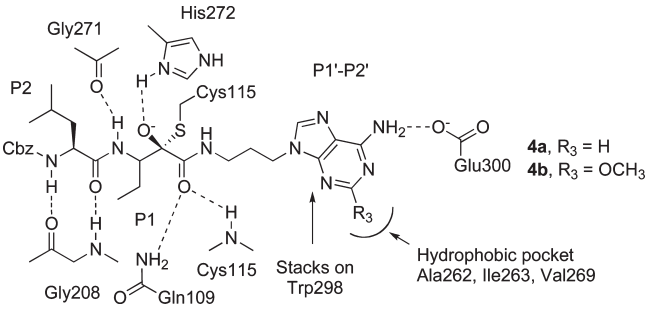

Figure 3. Proposed binding interactions of the peptidyl α-ketoamides **4a** and **4b** with the active site of calpain I.

when the nucleobase derivatives proved to be more potent inhibitors.

Nucleosides, nucleotides, and heterocyclic bases, the building blocks of RNA and DNA, are hydrophilic compounds and do not readily penetrate cell membranes by passive diffusion. Instead they are transported by several concentrative nucleoside transporters (CNT1, CNT2, and CNT3)[81] that are specific for the transport of different heterocyclic bases and nucleotides. Several of the compounds (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl (**4a**), Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin-3-yl (**4c**), Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl (**5a**), and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl (**5c**)) that we synthesized have nucleobases such as adenine and cytosine in the primed region to facilitate their recognition by BBB nucleoside transporter systems. Several drugs, such as nucleoside reverse transcriptase inhibitors that are used in the treatment of HIV infection, are transported into the CNS by these nucleoside transporters, while many protease inhibitors are not effectively transported.[82−86] Hence, attachment of structural features for recognition by brain transporter systems to calpain inhibitors appears to be a promising strategy for facilitating incorporation of these molecules into the brain. The effectiveness of this strategy for other tissue types has been demonstrated by Meier and co-workers who attached ketoamide calpain inhibitors to various muscle cell targeting capping groups to assist with accumulation of calpain inhibitors in muscle cells for the treatment of Duchenne muscular dystrophy and observed improved uptake of calpain inhibitors into muscle cells.[87]

Mechanism of Inhibition and Binding Mode. The mechanism of inhibition of calpain by α-ketoamides involves the formation of a reversible enzyme−inhibitor complex prior to attack of the active site cysteine residue (Cys115) on the keto carbonyl group of the α-ketoamides. This leads to the formation of a stable but reversible tetrahedral hemithioketal adduct (Figure 3) containing a hydrogen bond between the newly formed hydroxyl group of the tetrahedral adduct and the imidazole ring of His272.

Crystal structures of **4a** (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl) and **4d** (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) bound to the rat calpain I protease core (μI-II) have previously been reported by Campbell et al.[88] Figure 3 shows a schematic drawing of the interaction of **4a** with the active site of calpain. Important features in this structure are stacking of the adenine moiety of **4a** against a tryptophan (Trp298) in the catalytic site of calpain I, formation of a hydrogen bond between the amino group of adenine and the side chain of Glu300, formation of two hydrogen bonds between the carbonyl oxygen of the

Table 1. Inhibition of Calpain I, Calpain II, and Cathepsin B by Peptidyl α-Ketoamides[a]

		K_i (μM)			
	compd	Cal I	Cal II	Cat B	Cal I/Cat B
1	Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-morpholine[38]	0.150 ± 0.029	0.041	6.9	46
4a	Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl	0.053 ± 0.001	0.070 ± 0.010	0.80 ± 0.15	15.1
5a	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl	0.055 ± 0.009	0.068 ± 0.006	1.75 ± 0.18	31.8
4b	Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl	0.023 ± 0.006	0.077 ± 0.025	0.88 ± 0.02	38.3
5b	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl	0.041 ± 0.012	0.209 ± 0.029	2.34 ± 0	57.1
4c	Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin-3-yl	0.165 ± 0.020	1.14 ± 0.06	0.75 ± 0.06	4.54
5c	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl	0.48 ± 0.06	0.438 ± 0.079	0.44 ± 0.03	0.92
4d	Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl)	0.640 ± 0.137	0.286 ± 0.074	1.42 ± 0.34	2.22
5d	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl)	1.37 ± 0.126	6.36 ± 1.06	111 ± 17	81.0
5e	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$	0.226 ± 0.036	0.844 ± 0.317	75.5 ± 6.8	334
5f	Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$	0.711 ± 0.159	3.52 ± 0.54	475 ± 66	668

[a] Calpain assays were performed in 50 mM Tris HCl, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% CHAPS, pH 7.5, 10 mM DTT, 5 mM $CaCl_2$, and < 5% DMSO. Calpain I from porcine erythrocytes and calpain II from porcine kidney were used in the assays. The human liver cathepsin B assays were performed in 0.1 M $NaHPO_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer, and < 5% DMSO.

carboxamide with Gln109 and Cys115, and formation of hydrogen bonds between the inhibitor backbone and Gly208, Gly271 (Figure 3). Neither the stacking interaction with Trp298 nor hydrogen bond formation with Glu300 was observed between the piperazinyl ring of compound **4d** and the primed side region of the enzyme.

Interestingly, a hydrophobic pocket formed by Ala262, Ile263, and Val269 was observed in the crystal structure near the C2 carbon of the adenine moiety (Figure 3). To facilitate interactions with this hydrophobic pocket, we have synthesized several peptidyl α-ketoamides with a 2-methoxyadenine moiety in the primed region (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**4b**) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**5b**)).

The inhibitory potency of the new inhibitors toward calpain I, calpain II, and cathepsin B is shown in Table 1.

Calpain I Inhibition. As expected from the crystal structure, inhibitors with adenine (**4a**, **5a**) and 2-methoxyadenine (**4b**, **5b**) in the primed region were 3- to 7-fold more potent than our lead compound **1** (K_i = 150 nM). The best calpain I inhibitors were Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**4b**) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**5b**) with K_i values of 23 and 41 nM, respectively, while compounds Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenine-9-yl (**4a**) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenine-9-yl (**5a**) were slightly less potent than the 2-methoxyadenine derivatives but still have K_i values of 53 and 55 nM, respectively. The increased potency of the 2-methoxyadenine derivatives confirmed that the 2-methoxy group in these compounds is probably interacting with the hydrophobic pocket (Ala262, Ile263, and Val269) in the primed region (Figure 3). Compounds **4c** (K_i = 165 nM) and **5c** (K_i = 480 nM) with cytosine in the primed side (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin-3-yl, K_i = 165 nM; Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl, K_i = 480 nM) were 3- to 8-fold less potent than the adenine derivatives. However, the cytosine compounds are still very potent inhibitors, since they can also form a hydrogen bond with Glu300 and can stack on Trp298, although not as well as the adenine or 2-methoxyadenine derivatives. Among the compounds with nucleobases in the primed region, Abu in the P1 position is slightly favored over Phe.

Compounds containing 4-methylpiperazine (**4d**, K_i = 640 nM; **5d**, K_i = 1.37 μM) or dimethylamino alkyl groups (**5e**, K_i = 226 nM; **5f**, K_i = 711 nM) in the primed side were less potent than **1** and compounds with nucleobases in the primed side but are still reasonable inhibitors of calpain I. The decreased potency is probably due to the lack of the stacking interactions with Trp298 and the hydrogen bond with Glu300. Changing the amino acid in the P1 position from an Abu to a Phe resulted in a 100-fold increase in potency in compound **5e**, while decreasing the alkyl spacer by one methylene group in compound **5f** resulted in a 3-fold decrease in potency.

Calpain II Inhibition. In general, the inhibitors were more inhibitory toward calpain I but the order of reactivity of calpain II is similar to that of calpain I. Compounds with nucleobases (**4a**, **4b**, **5a**, and **5b**) in the primed region were more potent than those with dimethylaminoalkyl groups (**5e**,**f**). Compounds Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl (**4a**) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl (**5a**) were the most potent inhibitors of calpain II with K_i values of 70 and 68 nM, respectively. Introduction of the methoxy group to the C2 carbon of adenine did not significantly change the potency for Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**4b**) (K_i = 77 nM) but resulted in a 3-fold decrease in potency for Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl (**5b** (K_i = 209 nM). The cytosine derivatives Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin3-yl (**4c**) (K_i = 1.14 μM) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl (**5c**) (K_i = 438 nM) were less potent than the adenine and 2-methoxyadenine derivatives.

The 4-methylpiperazine derivatives Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) (**4d**) (K_i = 286 nM) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) (**5d**) (K_i = 6.36 μM) were less potent than the adenine derivatives **4a** and **5a**. The dimethylaminoalkyl analogues **5e** and **5f** have K_i values of 25.9 μM, 844 nM, and 3.52 μM, respectively. Again, replacing Abu with Phe in the P1 position resulted in a 30-fold increase in potency for compound **5e** and decreasing the alkyl spacer length by one methylene group decreased the potency 4-fold for Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$ (**5f**).

Cathepsin B Inhibition and Selectivity. In order to determine the selectivity of the new inhibitors, we measured inhibitory potency with cathepsin B. In general, the peptidyl α-ketoamides displayed lower affinity toward cathepsin B. The most selective calpain I inhibitors among the ones with nucleobases in the primed region were the 2-methoxyadenine derivatives. The inhibitor **4b** (Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl) with a K_i value of 23 nM

for calpain I is 3- and 38-fold poorer with calpain II and cathepsin B, respectively. The K_i of **5b** (Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl) is 41 nM, which makes it 5- and 57-fold more potent on calpain I than on calpain II and cathepsin B. The small hydrophobic pocket observed in the active site of calpain I is not present in cathepsin B; thus, both selectivity and increased potency toward calpain I have been obtained by the introduction of a small hydrophobic group on to the adenine ring to interact with the pocket.

Except for **4d** (K_i = 1.42 μM), most of the inhibitors with 4-methylpiperazine and dimethylaminoalkyl substituents in the primed region were poor inhibitors of cathepsin B (Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) (**5d**, K_i = 111 μM), Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$ (**5e**, K_i = 75.5 μM), Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$ (**5f**, K_i = 475 μM) except Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) with a K_i value of 1.42 μM). The most selective calpain I inhibitors among those with *N*-methylpiperazine and dimethylaminoalkyl substituents in the primed region were Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$ (**5e**) and Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$ (**5f**). Inhibitor **5e** (K_i = 0.226 μM toward calpain I) is 3- and 334-fold less potent on calpain II and cathepsin B, respectively, while inhibitor **5f** (K_i = 0.711 μM toward calpain I) is 5- and 668-fold less potent on calpain II and cathepsin B, respectively. The calpain inhibitors **5e** and **5f**, although less potent than several other inhibitors, have increased specificity for calpain and may be more useful in biological experiments.

Overall, cathepsin B was inhibited by the new peptidyl α-ketoamides, but good selectivity was obtained for calpain I and calpain II in α-ketoamides **5d**, **5e**, and **5f**. The cytosine derivative **5c** was equally potent with calpain I, calpain II, and cathepsin B.

Brain Permeability. In our initial studies demonstrating axonal protection in the animal model of peripheral neuropathy,[60] **1** was delivered continuously from a subcutaneous minidiffusion pump at a dose of **1** at 24 (mg/kg)/day. These studies showed that **1** (Figure 1) is an effective calpain inhibitor in vivo, and we therefore choose this dose to use in our animal studies. Liquid chromatography tandem mass spectrometric (LC−MS/MS) experiments were performed in order to determine the concentration of **1** in the brain, heart, kidney, liver, spinal cord, serum, peripheral (sciatic) nerve, and spleen of mice dosed with 24 mg of **1**/kg body weight via subcutaneous (sample cohort N = 2 mice), intravenous (N = 3 mice), or oral (N = 2 mice) administration and sacrificed after 1 (sc), 1 (iv), or 4 (oral) h, respectively (Table 2). The inhibitor **1** could not be detected in the brain but was present in the liver, heart, kidney, and spleen at levels of >0.5 μg/g of tissue after a single subcutaneous or intravenous dose, indicating that the bioavailability of **1** via subcutaneous or oral administration was good, although the inhibitor passed through the excretory system without penetrating the BBB.

Two of the newly synthesized compounds (**4a** and **4d**), which were designed to cross the BBB, were also analyzed in the brain of mice. For these studies, inhibitors (24 mg/kg body weight) were subcutaneously administered to mice sacrificed after 1 (N = 3), 2 (N = 3), 4 (N = 3), or 8 (N = 3) h (Table 3). The adenine compound **4a**, was only detected in quantifiable levels in 1 mouse out of 12. This mouse at the 8 h time point had a concentration of **4a** of 1.17 ± 0.01 μg/g tissue. The *N*-methylpiperazine derivative **4d** could be detected in the three mice at the 1 h time point but could not be quantitated with sufficient accuracy. After 2 h, **4d** could be detected and quantitated in one mouse at 1.14 ± 0.02 μg/g tissue. The concentrations observed for **4a** and **4d** are approximately 2 μM, which is several-fold higher than the K_i values for inhibition of calpain I and II, and thus, this is likely a therapeutically useful concentration.

The detection of the two compounds in the brains of several animals, but not all the animals, is certainly encouraging. However, this result only shows that the compounds are incorporated into the brain. This could result from either passive diffusion or active transport by one of the BBB barrier transporters. Future experiments should involve in vitro studies with various BBB transporters to determine if the compounds are indeed interacting with the transporters or are simply entering the brain by passive diffusion. It will also be necessary to develop a more sensitive and routine analytical method for **4a** and **4d** in tissue samples in order to test our hypothesis further and to validate our design strategy. The current analytical method was optimized for **1** and did not allow us to detect and quantitate **4a** and **4d** in the majority of animals dosed.

Table 2. Concentrations of **1** in Mouse Serum and Tissue Samples[a]

	concn of **1**, μg/(g of tissue)		
tissue	subcutaneous (N = 2; 1 h)	intravenous (N = 3; 1 h)	oral (N = 2; 4 h)
brain	ND	ND	ND
heart	0.541 ± 0.008	0.561 ± 0.036	NQ
kidney	0.936 ± 0.068	1.816 ± 0.161	NQ
liver	1.550 ± 0.068	0.943 ± 0.052	NQ
spinal cord	ND	ND	ND
serum	0.895 ± 0.196	NQ	NQ
peripheral nerve	NQ	0.333 ± 0.009[b]	NQ
spleen	1.380 ± 0.174	1.316 ± 0.067	NQ

[a] Concentration of **1** detected in the brain, heart, kidney, liver, spinal cord, serum, peripheral nerve, or spleen obtained from mice dosed with the inhibitor **1** at 24 mg/kg body weight. The mice received the drug by subcutaneous (N = 2, mice sacrificed after 1 h), intravenous (N = 3, mice sacrificed after 1 h), or oral (N = 2, mice sacrificed after 4 h) administration. A calibration curve was measure at varying doses of **1** in mouse plasma. The quantitation limit was 0.004 mg/mL plasma, and the detection limit was 0.001 mg/mL (1 ng/mL) plasma. No calibration curves were determined with individual tissues, and the quantitation limit is likely higher in tissue. The errors in the measurements were determined using multiple HPLC sample injections from the individual animals. ND = not detected. NQ = not quantifiable. [b] The value listed for the sciatic nerve with **1** was from three injections of a sample from one mouse dosed intravenously. The other two mice were NQ.

Table 3. Concentration of Calpain Inhibitors in the Mouse Brain after Subcutaneous Administration[a]

	μg/(g of tissue)			
compd	N = 3; 1 h	N = 3; 2 h	N = 3; 4 h	N = 3; 8 h
4a	ND	ND	ND	1.17 ± 0.01[b]
4d	NQ[c]	1.14 ± 0.02[b]	ND	ND

[a] Concentration of **4a** and **4d** detected in the brain obtained from mice dosed with the inhibitors at 24 mg/kg body weight. The mice received the drug by subcutaneous administration and were sacrificed after 1, 2, 4, and 4 h. A calibration curve was measured at varying doses of **4a** and **4d** in mouse plasma. The quantitation limit was 0.16 μg/mL plasma for **4a** and was 0.23 μg/mL plasma for **4d**. No calibration curves were determined for brain tissue. ND = not detected. NQ = not quantifiable. [b] The value listed is three injections of a sample from one mouse, and this group is N = 1. The two other mice were NQ. [c] The inhibitor was detected in all three mice.

Conclusions

We have shown that peptidyl α-ketoamides with the general structure of Cbz-L-Leu-D,L-AA-CONH-R, where R is a heterocyclic base, are effective inhibitors of the cysteine proteases calpain I, and II. It has been observed that Abu, which is a small hydrophobic residue, is slightly favored over the large hydrophobic residue Phe in the P1 position. It was observed that nucleobases were favored over dimethylaminoalkyl or methylpiperazine substituents in the primed region because of stacking interactions of the nucleobases with a Trp residue near the active site. Our hypothesis that introduction of a hydrophobic group on the adenine ring would facilitate interactions with the hydrophobic pocket observed in the crystal structure resulting in increased potency was verified experimentally for the new calpain inhibitors **4b** and **5b** with a 2-methoxyadenine group. In an effort to further extend permeability across the BBB to other structures, peptidyl α-ketoamides containing cytosine in the primed side were also synthesized but were less effective than the adenine derivatives.

Although our lead compound **1** was found in the peripheral nerve and other tissue, it was not detected in the brain. However, two compounds, **4a** and **4d**, have been detected at therapeutically useful concentrations in the brain of some mice after subcutaneous administration. Increased levels of calpain activity have been observed in a number of neurodegenerative diseases with brain involvement including Alzheimer's, Huntington's, and Parkinson's diseases and multiple sclerosis. Development of selective calpain inhibitors that can cross the BBB is required for the treatment of these diseases. Here, we have shown that peptidyl α-ketoamide calpain inhibitors potentially can be designed to cross the BBB and thus may be useful in the treatment of a variety of neurodegenerative diseases.

Experimental Section

Material and Methods. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The structures and purity of each target compound were confirmed by TLC, ^{1}H NMR, MS, HPLC analysis, and/or elemental analysis. TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). Electrospray ionization (ESI), fast-atom-bombardment (FAB), and high-resolution mass spectrometry (HRMS) were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. The purity of compounds **5a**, **4b**, **5b**, **4c**, and **5c** after purification was determined by elemental analysis and was higher than 95%. The elemental composition for each of these compounds is given in the Experimental Section for that compound. Elemental analyses were carried out by Atlantic Microlab Inc., Norcross, GA. The purity of **5d**, **5e**, and **5f** was determined by HPLC. The analysis was run on a Beckman Coulter HPLC running 32Karat, version 4.0, software. The Alltech/Applied Science C18 column used was 250 mm × 4.6 mm and packed with 5 μm Sperisorb ODS 2. The column was eluted with an isocratic mixture of 60% 0.1% TFA in acetonitrile and 40% 0.1% TFA in water. Detection was at 220 and 254 nm, and the area percent was measured using the 32Karat software in duplicate HPLC runs. Compound **5d** was 95−99% pure, **5e** was 89−91% pure, and **5f** was 96−99% pure. The synthesis of compounds **1**, **4a**, and **4d** has previously been reported.[36,38,88]

Animal Studies. All experiments involving animals were approved by the Emory University Institutional Animal Care and Use Committee. Animals were 7-week-old female C57BL/6 mice. Each experiment was done with cohorts of two to three animals per time point and dosed with 24 mg inhibitor/kg body weight, administered subcutaneously ($N = 2$ for **1**, $N = 3$ for **4a** and **4d**), intravenously ($N = 3$ for **1**), or orally via oral gavage ($N = 2$ for **1**). For compound **1**, mice dosed subcutaneously, intravenously, or orally were sacrificed after 1, 1, or 4 h, respectively. For compounds **4a** and **4d**, mice ($N = 3$) were sacrificed 1, 2, 4, or 8 h after the dose was administered. At the designated time, each animal was perfused with buffered saline at 37 °C to clear all blood vessels and was sacrificed. Serum and extracted tissue samples were frozen in liquid nitrogen and stored at −80 °C until analyzed by LC−MS/MS.

LC−MS/MS Assays. For the measurement of plasma pharmacokinetics, tissue distribution, and permeability into the nervous system of **1**, **4a**, and **4d**, a LC−MS/MS assay was developed using another of our calpain inhibitor compounds, Cbz-Leu-Nva-CONH-$(CH_2)_3$-morpholine (ZLAK74), as an internal standard. The internal standard was added to serum or tissues early in the sample preparation procedure to compensate for incomplete analyte extraction. All drug standards and tissues were stored at −80 °C until needed. Control tissues from undosed mice were used to prepare calibration standards with concentrations of 0.10, 0.30, 0.50, and 1.00 μM for inhibitor **1**. The calibration standards for **4a** and **4d** where prepared at identical concentration in mouse plasma. Sample preparation for the spiked control tissues (or plasma) and the sample tissues was identical and based on a protocol reported by Guo and coworkers.[89] First, the tissues were homogenized with water in a 5% (weight/volume) ratio (i.e., 5 g tissue/mL water) and 100 μL aliquots of each homogenate were pipetted into centrifuge tubes and spiked with 1.00 μM Cbz-Leu-Nva-CONH-$(CH_2)_3$-morpholine as an internal standard. The mixture was sonicated for 10 min to ensure homogeneity in the sample, and an amount of 300 μL of 99.9:0.1 v/v acetonitrile/formic acid was added to precipitate proteins. The sample was briefly vortexed and sonicated for 15 min followed by centrifugation at 13000*g* for 30 min. The supernatant was removed and evaporated at 45 °C for 3 h, and the residue was reconstituted with 100 μL of 30:69.9:0.1 v/v acetonitrile/water/formic acid. The final solution was filtered using a 0.45 μm syringe filter (Acrodisc, Pall) and analyzed by LC−MS/MS.

All measurements were carried out in an Agilent HPLC 1100 system coupled to a ThermoFinnigan LCQ Deca XP+ ion trap mass spectrometer using an ESI source operating in positive ion mode. For experiments with compound **1**, an analytical Zorbax Extend C18 column (1.0 mm × 150 mm, 5 μm particles, 80 Å pore size, Agilent) was used with the following binary gradient program: 0.0 min, 30% B; 0.5 min, 30% B; 0.7 min, 85% B; 2.5 min, 85% B; 2.70 min, 30% B; 5.5 min, 30% B. An analytical Symmetry Shield reverse-phase C18 column (1.0 mm × 150 mm, 3.5 μm particles, 100 Å pore size; Waters) preceded by a Zorbax RX-C18 guard column (4.6 mm × 12.5 mm, 5.0 μm particles, 2 μm pore size; Agilent) was used for experiments with compounds **4a** and **4d**. For separations using this column, the binary solvent gradient program used was as follows: 0.0 min, 30% B; 0.5 min, 30% B; 0.7 min, 100% B; 4.5 min, 100% B; 5.0 min −30% B; 6.0 min, 30% B. The mobile phases used for all separations were as follows: A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile. The flow rate was 80 μL min^{-1}, with an injection volume of 15 μL.

The mass spectrometer was operated in multiple reaction monitoring mode using the following precursor → fragment transitions, **1**: m/z 505.2 → 443.2. Cbz-Leu-Nva-CONH-$(CH_2)_3$-morpholine (internal standard): m/z 519.2 → 457.2. **4a**: m/z 553.2 → 509.2. **4d**: m/z 518.3 → 410.3. The following settings were used: ESI needle voltage +4.0 kV; sheath gas 15 arbitrary units (~0.6 L min^{-1}); capillary temperature 275 °C; capillary voltage 34 V (**1**), 28 V (**4a**), 18 V (**4d**); capillary skimmer voltage 25 V (**1**), 55 V (**4a**), 45 V (**4d**). For all experiments, the mass analyzer was set with the automatic gain control on 1×10^7 with

2 microscans, 400 μs max injection time, 1.2 Da mass selection window, and 34% normalized collision energy. After acquisition, peak areas for the chromatographic peaks present in the extracted ion chromatograms for the internal standard and the compound of interest were determined using the mass spectrometer software Xcalibur 2.0 (Thermo) with the Genesis peak detection algorithm (15 smoothing points, signal-to-noise ratio threshold of 3.0). The areas for the compound of interest and the internal standard were exported to Excel, and the ratio of the peak area of the compound of interest to peak area of Cbz-Leu-Nva-CONH-$(CH_2)_3$-morpholine (internal standard) was calculated. The amount of compound present in each sample was determined by correlating the area ratio to a concentration using the calibration curves obtained during each experiment.

Calpain I and Calpain II Assays. The fluorogenic substrate Suc-Leu-Tyr-AMC was obtained from Bachem. Calpain I from porcine erythrocytes and calpain II from porcine kidney were purchased from Calbiochem. The fluorescence was monitored using a Tecan Spectrafluor microplate reader. AMC was used as the calibration standard, and the calibration curve was plotted against RFU for different concentrations of AMC within the range 5−0.08 μM. Inhibitor stock solutions were prepared in DMSO and kept at 4 °C prior to use. Calpain assays were performed in 50 mM Tris HCl, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% CHAPS, pH 7.5, 10 mM DTT, 5 mM $CaCl_2$, and three different substrate (Suc-Leu-Tyr-AMC) concentrations (0.8, 0.4, 0.2 μM). A 10 μL aliquot of DMSO (control) or inhibitor solution in DMSO (DMSO content of < 5%) was added to 200 μL of buffer. The reaction was initiated by adding a 2 μL aliquot of enzyme (with a final concentration of 10 nM) to the well. The reaction was monitored by the release of 7-amino-4-methylcoumarin (λ_{ex} = 360 nm, λ_{em} = 465 nm). The total volume in the reaction well was 212 μL, and controls were run every hour. Velocities were determined at room temperature (RT) at five or more concentrations of inhibitor and at three fixed concentrations of substrate. A plot of $1/v$ versus [I] gave intersecting lines with a correlation coefficient of ≥0.95. K_i values were determined by Dixon plots.[90]

Cathepsin B Assay. The fluorogenic substrate Cbz-Arg-Arg-AMC was obtained from Bachem. Cathepsin B from human liver was purchased from Calbiochem. The fluorescence was monitored and calibrated using the method reported for calpain I and II above. Inhibitor stock solutions were prepared in DMSO and kept at 4 °C prior to use. The cathepsin B assay was performed in 0.1 M $NaHPO_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer, and three different substrate (Cbz-Arg-Arg-AMC) concentrations (0.5, 0.2, 0.1 μM). A 10 μL aliquot of DMSO (control) or inhibitor solution in DMSO (DMSO content < 5%) was added to 200 μL of buffer. The reaction was initiated by adding 5 μL of activated enzyme (with a final concentration of 0.4 μM) to the well. The enzyme was activated by the addition of cathepsin B kinetic buffer (267 μL) and 0.1 M DTT (3 μL) to the enzyme stock solution (30 μL). The reaction was monitored by the release of 7-amino-4-methylcoumarin (λ_{ex} = 360 nm, λ_{em} = 465 nm). The total volume in the reaction well was 215 μL, and controls were run every hour. Velocities and K_i values were determined using the aforementioned method for calpain I and II.

Statistical Analysis. The data in Table 1 for calpain I, calpain II, and cathepsin B were analyzed using the VassarStats Web site for statistical computation. This Web site was developed by Professor Richard Lowry. Specifically a one-way ANOVA with a post hoc Tukey HSD (honestly significant differences) test was performed. Further details and the comparisons are given in the Supporting Information. In general, inhibition constants for calpain I, calpain II, and and cathepsin B that differed by less than a factor of approximately 2 were considered to be nonsignificant in this analysis. In general, differences in potency between calpain I and calpain II for each compound were nonsignificant with three exceptions (**4b**, **5b**, and **4c**), while the differences between calpain I and cathepsin B and between calpain II and cathepsin II were always significant with one exception (**5c**).

General Procedure for the Synthesis of Dipeptide α-Ketoesters. The synthesis of the dipeptide acids Cbz-Leu-Abu-OH and Cbz-Leu-Phe-OH are given in the Supporting Information. The dipeptide acid (1 equiv) was dissolved in dry THF, and 4-dimethylaminopyridine (0.05 equiv), pyridine (3 equiv), and ethyl oxalyl chloride (2.1 equiv) were added sequentially. The resulting mixture was stirred at reflux temperature for 4 h. After removal of the heat source, 1 M HCl (50 mL) was added to the brown solution. The mixture was extracted with ethyl acetate (2 × 100 mL). The combined extract was washed with 100 mL of saturated NaCl, dried over $MgSO_4$ overnight, and filtered. Ethyl acetate was removed from the filtrate to give a mixture of products containing the dipeptide enol ester. The mixture of products was dissolved in 20 mL of absolute ethanol and stirred in an ice bath. Triethylamine (1 equiv) was added, and the mixture was stirred for 1 h at RT. Solvent was removed from the final mixture using a rotary evaporator. The crude oil was subjected to column chromatography to give the dipeptidyl α-ketoester. These compounds have previously been reported using a synthetic scheme with more steps.[36,38]

Cbz-Leu-D,L-Abu-COOEt: light-yellow oil, 76% yield. ^{1}H NMR ($CDCl_3$): 0.86−0.93 (m, 9H, 2 × Leu-CH_3 and Abu-CH_3), 1.26−1.37 (m, 4H, CH_3 and Leu-CH), 1.49−1.98 (m, 4H, Abu-CH_2, Leu-CH_2 and CH_3), 4.23−4.38 (m, 2H, 2×α-H), 4.42−4.46 (m, 2H, CH_2), 5.03−5.13 (m, 2H, Cbz), 5.67−5.73 (m, 1H, NH), 7.21−7.32 (m, 6H, Ph and NH). HRMS (FAB) calcd for $C_{21}H_{31}N_2O_6$: 407.2182. Observed m/z 407.2178 ($[M + H]^+$).

Cbz-Leu-D,L-Phe-COOEt: light-yellow oil, 68% yield. ^{1}H NMR ($CDCl_3$): 0.79−0.90 (m, 6H, 2 × Leu-CH_3), 1.22−1.61 (m, 6H, Leu-CH_2, CH_3 and Leu-CH), 2.93−3.07 (m, 1H, CH), 3.19−3.28 (m, 1H, CH), 4.14−4.33 (m, 4H, CH_2 and 2 × α-H), 5.08 (d, 2H, Cbz), 5.23−5.33 (m, 1H, NH), 6.77−6.84 (m, 1H, NH), 7.12−7.29 (m, 5H, Ph), 7.33 (s, 5H, Ph). HRMS (FAB) calcd for $C_{26}H_{33}N_2O_6$: 469.2339. Observed m/z 469.2337 ($[M + H]^+$).

General Procedure for the Synthesis of Dipeptidyl α-Ketoacids. Dipeptidyl α-ketoesters (1 equiv) were dissolved in ethanol, and 1 M NaOH solution (1.1 equiv) was added in portions while stirring in an ice bath. The resulting mixture was stirred at RT for an hour and extracted with anhydrous ether (4 × 30 mL). The aqueous layer was acidified to pH 4 with 2 M HCl in an ice bath and extracted with diethyl ether (Et_2O, 2 × 50 mL). The combined ether extract was washed with saturated NaCl, dried over $MgSO_4$ overnight, and filtered. Ether was removed from the filtrate by evaporation, and the product was dried under reduced pressure. These compounds have previously been reported using a synthetic scheme with more steps.[36,38]

Cbz-Leu-D,L-Abu-COOH (2): pale-yellow hygroscopic flakes, 96% yield. ^{1}H NMR ($CDCl_3$): 0.91 (d, 9H, Abu-CH_3 and 2 × Leu-CH_3), 1.47−1.75 (m, 5H, Leu-CH_2, Abu-CH_2, Leu-CH), 4.13−4.35 (m, 2H, 2 × α-H), 5.04−5.13 (m, 3H, Cbz and NH), 7.32 (s, 5H, Ph), 8.35−8.41 (d, 1H, NH). HRMS (FAB) calcd for $C_{19}H_{27}N_2O_6$: 379.1869. Observed m/z 379.1870 ($[M + H]^+$).

Cbz-Leu-D,L-Phe-COOH (3): pale-yellow hygroscopic flakes, 89% yield. ^{1}H NMR ($CDCl_3$): 0.77−0.86 (m, 6H, 2 × Leu-CH_3), 1.09−1.56 (m, 3H, Leu-CH_2 and Leu-CH), 2.49−2.51 (m, 1H, CH), 2.75−2.91 (m, 1H, CH), 4.01−4.08 (m, 2H, 2 × α-H), 4.89−5.06 (m, 3H, Cbz and NH), 7.18−7.40 (m, 10H, 2 × Ph), 8.49 (t, 1H, NH). HRMS (FAB) calcd for $C_{24}H_{29}N_2O_6$: 441.2026. Observed m/z 441.2025 ($[M + H]^+$).

Synthesis of 9-(3-Aminopropyl)adenine.[66] The synthesis has been previously reported and experimental details are given in Supporting Information.

Synthesis of 9-(3-Aminopropyl)-2-methoxyadenine. A mixture of 2-chloroadenine (1 equiv) and sodium methoxide (7.5 equiv) in anhydrous methanol (50 mL) was sealed in pressure vessel. The reaction mixture was held at an internal temperature of 100 °C for 24 h before cooling to RT. Once cooled, the pressure

vessel was opened and the suspension was diluted with water (50 mL). The resulting solution was evaporated under reduced pressure to give a final volume of 70 mL; water (30 mL) was added to this solution to give a final volume of 100 mL. The solution was transferred to a three-neck flask equipped with a stirrer, thermometer, and pH meter. The solution was heated to 60 °C (internal temperature), and 50% aqueous HCl was added to adjust the pH to 9.5. The resulting suspension was stirred at 60 °C for 1 h, cooled slowly to RT, and stirred for 16 h. The suspension was filtered, and the filter cake was washed with water (10 mL) and methanol (2 × 10 mL). The solid was dried under vacuum to give 2-methoxyadenine in 70% yield. ^{1}H NMR (DMSO-d_6): 3.76 (s, 3H, OCH_3), 7.12 (s, 2H, NH_2), 7.86 (s, 1H, CH).

A mixture of 2-methoxyadenine (1 equiv), 1-bromo-3-chloropropane (4.3 equiv), and potassium carbonate (2.35) in DMF (200 mL) was stirred at RT under argon for 4 days, filtered, and evaporated to dryness. The crude product was purified by column chromatography and gave 9-(3-chloropropyl)-2-methoxyadenine in 66% yield. MS (ESI) m/z 241.9 ([M + H]$^+$).

A mixture of 9-(3-chloropropyl)-2-methoxyadenine (1 equiv) and sodium azide (3 equiv) in DMF was stirred at 80 °C for 24 h, cooled to RT, and filtered. The crude product was purified by column chromatography to give 9-(3-azidopropyl)-2-methoxyadenine as a white crystalline solid in 74% yield. ^{1}H NMR (DMSO-d_6): 1.99−2.06 (m, 2H, CH_2), 3.33−3.37 (m, 2H, CH_2), 3.80 (s, 3H, OCH_3), 4.10 (t, 2H, CH_2), 7.21 (s, 2H, NH_2), 7.92 (s, 1H, CH). MS (ESI) m/z 249.0 ([M + H]$^+$).

A mixture of 9-(3-azidopropyl)-2-methoxyadenine and 5% palladium on carbon in MeOH was reacted with hydrogen gas at RT for 20 h. The catalyst was removed by filtration and the solvent removed to give 9-(3-aminopropyl)-2-methoxyadenine as a white solid in 75% yield. ^{1}H NMR (DMSO-d_6): 1.82−1.85 (m, 2H, CH_2), 2.46−2.49 (m, 2H, CH_2), 3.03 (s, 2H, NH_2), 3.79 (s, 3H, OCH_3), 4.09 (t, 2H, CH_2), 7.20 (s, 2H, NH_2), 7.92 (s, 1H, CH). MS (ESI) m/z 223.2 ([M + H]$^+$).

Synthesis of 1-(3-Aminopropyl)cytosine.[68] The synthesis has been previously reported, and experimental details are given in Supporting Information.

Synthesis of 1-(3-Aminopropyl)-4-methylpiperazine.[69] The synthesis has been previously reported, and experimental details are given in Supporting Information.

General Procedure for the Synthesis of Target Peptide α-Ketoamides by the HOBt and EDC Coupling Method. HOBt (1.5 equiv) was added to a stirred solution of the dipeptidyl α-ketoacid (1.5 equiv) in DMF at −10 °C, followed by addition of the heterocyclic amine (1 equiv) and EDC (1.5 equiv). The mixture was allowed to react for 16 h at RT. DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl before being dried over $MgSO_4$ and concentrated. Column chromatography on silica gel was used to purify the peptidyl α-ketoamides.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(6-amino-9*H*-purin-9-yl)propyl)-2-oxopentanamide (4a, Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-adenin-9-yl). This ketoamide has previously been reported,[88] and characterization data are shown in the Supporting Information.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(6-amino-9*H*-purin-9-yl)propyl)-2-oxophenylbutanamide (5a, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl). The ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-adenin-9-yl was obtained from 9-(3-aminopropyl)adenine and the ketoacid Cbz-Leu-D,L-Phe-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent, then recrystallized from EtOAc/hexane to give a white solid (21% yield). ^{1}H NMR (DMSO-d_6): 0.69−0.86 (m, 9H, 2 × Leu-CH_3 and Abu-CH_3), 1.15−1.36 (m, 5H, 2 × CH_2 and CH), 1.92−2.00 (m, 2H, CH_2), 3.04−3.15 (m, 4H, 2 × CH_2), 4.07−4.14 (m, 2H, CH_2 and 2 × α-H), 4.95−5.01 (m, 2H, Cbz), 5.18 (s, 1H, NH), 7.12−7.40 (m, 12H, 2 × Ph and NH_2), 8.08−8.14 (m, 2H, 2 × adenine-CH), 8.33−8.39 (d, 1H, NH), 8.83−8.89 (t, 1H, NH). HRMS (FAB) calcd for $C_{32}H_{39}N_8O_5$: 615.3043. Observed m/z 615.3094 ([M + H]$^+$). Anal. ($C_{32}H_{38}N_8O_5 \cdot 0.75H_2O$) C, H, N.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(6-amino-2-methoxy-9*H*-purin-9-yl)propyl)-2-oxopentanamide (4b, Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl). The ketoamide product Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl was obtained from 9-(3-aminopropyl)-2-methoxyadenine and the ketoacid Cbz-Leu-D,L-Abu-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent, then recrystallized from EtOAc/hexane to give a yellowish white solid (26% yield). ^{1}H NMR (DMSO-d_6): 0.72−0.93 (m, 9H, 2 × CH_3 of Leu and CH_3 of Abu), 1.37−1.60 (m, 4H, CH_2 of Leu and CH_2 of Abu), 1.76 (m, 1H, CH of Leu), 1.93−1.97 (m, 2H, CH_2), 3.09 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 3.98−4.14 (m, 3H, CH_2 and α-H), 4.84 (m, 1H, α-H), 4.99 (s, 2H, Cbz), 7.20 (s, 2H, NH_2), 7.28−7.40 (m, 6H, Ph and NH), 7.93 (s, 1H, CH of adenine), 8.24−8.31 (m, 1H, NH), 8.77 (m, 1H, NH). HRMS (FAB) calcd for $C_{28}H_{41}N_6O_6$: 583.2993. Observed m/z 583.2900 ([M + H]$^+$). Anal. ($C_{28}H_{39}N_8O_6 \cdot 0.55H_2O$) C, H, N.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(6-amino-2-methoxy-9*H*-purin-9-yl)propyl)-2-oxophenylbutanamide (5b, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl). The ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl was obtained from 9-(3-aminopropyl)-2-methoxyadenine and the ketoacid Cbz-Leu-D,L-Phe-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent, then recrystallized from EtOAc/hexane to give a yellow solid (24% yield). ^{1}H NMR (DMSO-d_6): 0.73−0.75 (d, 6H, 2 × CH_3 of Leu), 1.11−1.35 (m, 4H, CH_2 Leu and CH_2 of Phe), 1.54 (m, 1H CH of Leu), 1.95−1.98 (m, 2H, CH_2), 3.09−3.12 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 4.03 (m, 3H, CH_2 and α-H), 4.97 (s, 2H, Cbz), 5.17 (m, 1H, α-H), 7.20−7.33 (m, 12H, 2 × Ph and NH_2), 7.93 (d, 1H, CH of adenine), 8.38 (d, 1H, NH), 8.84 (m, 1H, NH). HRMS (FAB) calcd for $C_{33}H_{41}N_6O_6$: 645.3149. Observed m/z 645.3067 ([M + H]$^+$). Anal. ($C_{33}H_{40}N_8O_6 \cdot 0.25H_2O$) C, H, N.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propyl))-2-oxopentanamide (4c, Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin-3-yl). The ketoamide product Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-cytosin-3-yl was obtained from 1-(3-aminopropyl)cytosine and the ketoacid Cbz-Leu-D,L-Abu-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent, then recrystallized from EtOAc/hexane to give a yellowish white solid (11% yield). ^{1}H NMR (DMSO-d_6): 0.72−0.95 (m, 9H, 2 × CH_3 of Leu and CH_3 of Abu), 1.41−1.73 (m, 5H, CH_2 of Leu, CH_2 of Abu and CH of Leu), 2.13−2.19 (m, 2H, CH_2), 3.07 (m, 2H, CH_2), 3.56 (m, 1H, α-H), 3.93−4.10 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 5.60 (d, 1H, CH of cytosine), 6.97 (d, 2H, NH_2), 7.32−7.44 (m, 6H, Ph and NH), 7.68 (d, 1H, CH of cytosine), 8.22−8.28 (m, 1H, NH), 8.73 (m, 1H, NH). HRMS (FAB) calcd for $C_{26}H_{37}N_6O_6$: 529.2775. Observed m/z 529.2781 ([M + H]$^+$). Anal. ($C_{26}H_{36}N_6O_6 \cdot 1$EtOAc) C, H, N.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(4-amino-2-oxopyrimidin-1(2*H*)-yl)propyl))-2-oxophenylbutanamide (5c, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl). The ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-cytosin-3-yl was obtained from 1-(3-aminopropyl)cytosine and the ketoacid Cbz-Leu-D,L-Phe-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent, then recrystallized from EtOAc/hexane to give a yellow solid (12% yield). ^{1}H NMR (DMSO-d_6): 0.74−0.76 (d, 6H, 2 × CH_3 of Leu), 1.11−1.37 (m, 4H, CH_2 Leu and CH_2 of Phe), 1.59 (m, 1H CH of Leu), 2.11−2.15 (m, 2H, CH_2), 3.01−3.10 (m, 2H, CH_2), 3.82−4.03 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 5.18 (m, 1H, NH),

5.61 (d, 1H, CH of cytosine), 6.97 (d, 2H, NH_2), 7.13−7.57 (m, 11H, 2 × Ph and CH of cytosine), 8.36 (d, 1H, NH), 8.80 (m, 1H, NH). HRMS (FAB) calcd for $C_{31}H_{39}N_6O_6$: 591.2886. Observed m/z 591.2852 ($[M + H]^+$). Anal. ($C_{31}H_{38}N_6O_6 \cdot 1EtOAc$) C, H, N.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(4-methylpiperazin-1-yl)propyl)-2-oxopentanamide (4d, Cbz-Leu-D,L-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl). This ketoamide has previously been reported,[88] and characterization data are shown in the Supporting Information.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(4-methylpiperazin-1-yl)propyl)-2-oxophenylbutanamide (5d, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl)). The dipeptide ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl was obtained from Cbz-Leu-D,L-Phe-COOH and 1-methyl-4-(3-aminopropyl)piperazine using the EDC/HOBt coupling method and purified twice by column chromatography on silica gel with 85:15 CH_2Cl_2/MeOH as the eluent to give a yellow semisolid in 10% yield. ^{1}H NMR ($CDCl_3$): 0.81 (m, 6H, CH_3 of Leu), 1.40−1.60 (m, 5H, CH_2 and CH), 2.28 (s, 6H, CH_3), 3.00 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 4.05 (m, 2H, CH_2), 4.50 (b, 1H, α-H), 5.02 (m, 3H, Cbz and α-H), 6.70 (b, 1H, NH), 7.05−7.30 (m, 7H, Ph and NH). The purity was 95−99% by HPLC using either 220 or 254 nm detection.

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(3-(dimethylamino)-propyl)-2-oxophenylbutanamide (5e, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-$N(CH_3)_2$). The dipeptide ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_3$-$N(CH_3)_2$ was obtained from Cbz-Leu-D,L-Phe-COOH and $(CH_3)_2N(CH_2)_3NH_2$ using the EDC/HOBt coupling method. Purification by column chromatography twice on silica gel with 80:20 CH_2Cl_2/MeOH as the eluent provided a yellow semisolid in 10% yield. ^{1}H NMR ($CDCl_3$): 0.84 (m, 6H, CH_3 of Leu), 1.50−1.80 (m, 5H, CH_2 and CH), 2.12 and 2.19 (d, 6H, CH_3), 3.00 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 4.15 (m, 2H, CH_2), 4.50 (b, 1H, α-H), 5.10 (m, 3H, Cbz and α-H), 6.82 (b, 1H, NH), 7.05−7.30 (m, 6H, Ph and NH), 7.40 (b, 1H, NH). The purity was 89−91% by HPLC using either 220 or 254 nm detection. The impurity is likely the dipeptide Cbz-Leu-Phe-NH-$(CH_2)_3$-$N(CH_3)_2$. HRMS (FAB) for $C_{29}H_{41}N_4O_5$: m/z 525.3077 ($[M + H]^+$).

3-(Benzyloxycarbonyl-L-leucylamino)-*N*-(2-(dimethylamino)-ethyl)-2-oxopentanamide (5f, Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-$N(CH_3)_2$). The dipeptide ketoamide product Cbz-Leu-D,L-Phe-CONH-$(CH_2)_2$-$N(CH_3)_2$ was obtained from Cbz-Leu-D,L-Phe-COOH and $(CH_3)_2N(CH_2)_2NH_2$ using the EDC/HOBt coupling method. Purification twice by column chromatography on silica gel with 80:20 CH_2Cl_2/MeOH as the eluent gave a yellow semisolid in 7% yield. ^{1}H NMR ($CDCl_3$): 0.85 (m, 6H, CH_3 of Leu), 1.50−1.70 (m, 3H, CH_2 and CH), 2.46 (s, 6H, CH_3), 3.00 (m, 2H, CH_2), 4.20 (m, 2H, CH_2), 4.31 (m, 2H, CH_2), 4.90 (b, 1H, α-H), 5.00 (m, 3H, Cbz and α-H), 6.20 (b, 1H, NH), 7.00−7.30 (m, 7H, Ph and NH). The purity was 96−99% by HPLC using either 220 or 254 nm detection. HRMS (FAB) for $C_{28}H_{39}N_4O_5$: m/z 511.3099 ($[M + H]^+$).

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Supporting Information Available: Synthesis of precursor dipeptides and amines; characterization of previously reported peptidyl ketoamides; statistical analysis of the results in Table 1 using a one-way ANOVA with a post hoc Tukey HSD (honestly significant differences) test. This material is available free of charge via the Internet at http://pubs.acs.org.

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