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Design, Synthesis, and Structure−Activity Relationship Studies of a Potent PACE4 Inhibitor

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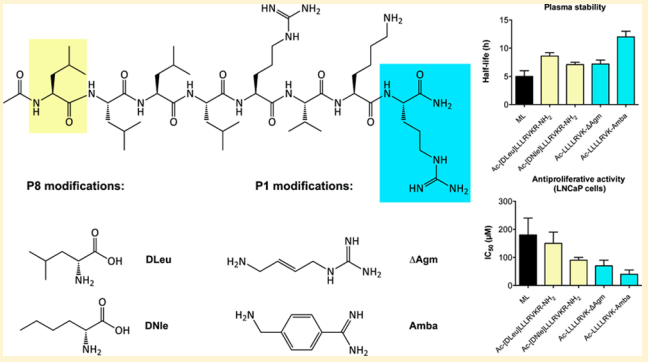
Supporting Information

ABSTRACT: PACE4 plays an important role in the progression of prostate cancer and is an attractive target for the development of novel inhibitor-based tumor therapies. We previously reported the design and synthesis of a novel, potent, and relatively selective PACE4 inhibitor known as a Multi-Leu (ML) peptide. In the present work, we examined the ML peptide through detailed structure−activity relationship studies. A variety of ML-peptide analogues modified at the P8−P5 positions with leucine isomers (Nle, DLeu, and DNle) or substituted at the P1 position with arginine mimetics were tested for their inhibitory activity, specificity, stability, and antiproliferative effect. By incorporating D isomers at the P8 position or a decarboxylated arginine mimetic, we obtained analogues with an improved stability profile and excellent antiproliferative properties. The DLeu or DNle residue also has improved specificity toward PACE4, whereas specificity was reduced for a peptide modified with the arginine mimetic, such as 4-amidinobenzylamide.



INTRODUCTION

The proprotein convertases (PCs) are a family of Ca^{2+}-dependent serine endoproteases involved in many physiological processes, including the activation of a wide array of regulatory proteins (e.g., receptor pro-forms, hormones, growth factors, zymogens, or cell membrane proteins).[1] Nine human PCs have been identified, namely, furin, PC1/3, PC2, PACE4, PC4, PC5/6, PC7, PCSK9, and SKI-1/S1P. The first seven members of this family have a cleavage preference for substrates containing multibasic cleavage motifs, for which a consensus sequence has been established, namely, Arg-X-Arg/Lys-Arg↓.[2] The other two enzymes exhibit different substrate specificities. SKI-1/S1P cleaves its substrates at nonbasic residues,[3] whereas PCSK9 cleaves itself once and acts in a nonenzymatic fashion as a binding protein to the low-density lipoprotein receptor, thus regulating blood cholesterol levels.[4] In addition to their normal physiological function, there is a large body of literature describing the implication of PCs in numerous pathological states, for example, viral and bacterial infections, tumorigenesis, neurodegenerative disorders, diabetes, and dyslipidemia.[5] Therefore, PCs have emerged as potential targets for the development of novel therapeutics.[5−7]

A wide variety of PC inhibitors have been reported, and among these are proteins,[8] peptides,[9,10] peptidomimetics,[11,12] small molecules,[13] antisense RNA,[14] and monoclonal antibodies for PCSK9.[15] The earliest studies included the use of furin inhibitors for the treatment of cancer as well as viral and pathogenic infections.[5,7] As examples, the injection of cancer cells expressing the protein-based furin inhibitor α1-Antitrypsin Portland demonstrated decreased tumorigenesis and metastasis in transgenic mice,[16] and the irreversible synthetic inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-CMK) has been shown to prevent tumor growth through the inhibition of matrix metalloproteinase 2 processing.[17] However, these inhibitors are not expected to become therapeutic agents because of their size, toxicity, lack of specificity, and stability.[5,12,18] Therefore, they have been mainly used to establish proof of concept for the role of PCs in various

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diseases. In contrast, peptidomimetic furin inhibitors appear to be promising compounds because of their antiviral activity and their protective effect against bacterial toxins, thereby offering possibilities to generate more efficient treatments for infectious diseases.[11,12] Moreover, on the basis of the crystal structure of furin, several nonpeptide small molecules have been developed;[13,19,20] however, among them, only tetrabasic 2,5-dideoxystreptamine derivatives exhibit potent activity against furin, with inhibition constants (K_i) values <10 nM, and have a protective effect against intoxication of macrophages by anthrax protective antigen.[13] Despite their excellent potency, none of these compounds have reached the clinical development stage so far.

Our recent studies revealed that another PC, namely, PACE4, is a viable therapeutic target for prostate cancer (PCa). We showed that PACE4 is overexpressed in PCa and plays a major role in the progression of the disease to more aggressive forms. In addition, we demonstrated that inhibition of this PC results in a decreased cellular proliferation rate and reduced tumorigenic growth in nude mice.[21] Moreover, our studies also showed that PACE4 has an essential role that is not compensated by other cancer-associated PCs, such as furin or PC7.[22] On the basis of these findings, we proposed that PACE4 is a novel target for the management of PCa and that compounds that target PACE4 would make good PCa therapeutic agents. However, the high degree of sequence and structural homology between the catalytic domains of PCs complicates the development of PACE4-specific inhibitors. Nevertheless, our group has successfully generated a potent and relatively selective PACE4 inhibitor (20-fold specificity over furin) known as the Multi-Leu (ML) peptide.[23] This peptide inhibitor exhibits potent antiproliferative effects on PCa cell lines DU145 and LNCaP. The pharmacological profile of the ML peptide makes it a promising lead compound for the development of novel anti-PCa agents.

Peptides have a number of advantages over small organic molecules, proteins, and antibodies as drug candidates because of their high biological activity, specificity, and low toxicity. However, the utility of peptides is limited by their poor metabolic stability, which results in a short duration of in vivo activity and low bioavailability. In the present study, we set out to overcome these limitations by introducing various chemical modifications into the structure of the ML-peptide inhibitor. Our strategy consisted of identifying the amino acids that could be substituted in combination with an understanding of the specificity and stability profile of the ML peptide. We used a peptidomimetic strategy that involves unnatural or D amino acid substitutions and arginine mimetics to create more stable and potent analogues of the ML peptide. The best compounds were then tested for their continued efficacy for anti-PCa activity.

RESULTS

Synthesis. Our peptidomimetic strategy is presented in Figure 1. On the basis of our previous results showing that the leucine core plays a key role in the specificity of a ML-peptide inhibitor for PACE4,[23] only leucine isomers (unusual or/and D amino acids) were used. We designed two series of ML-peptide analogues modified by single or multiple amino acid substitutions with L-norleucine or D-leucine/D-norleucine at the P8, P7, P6, and P5 positions. A recent study by Becker et al.[11] demonstrated that the introduction of a decarboxylated and conformationally restricted arginine mimetic in the P1

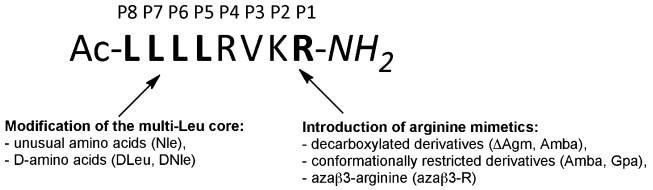

Figure 1. Proposed modifications of the Multi-Leu peptide inhibitor.

position of a furin inhibitor significantly improved its potency. Therefore, in a second approach, the P1 position of the ML-peptide inhibitor was replaced by arginine mimetics with similar chemical character (Figure 2). In addition, we designed a

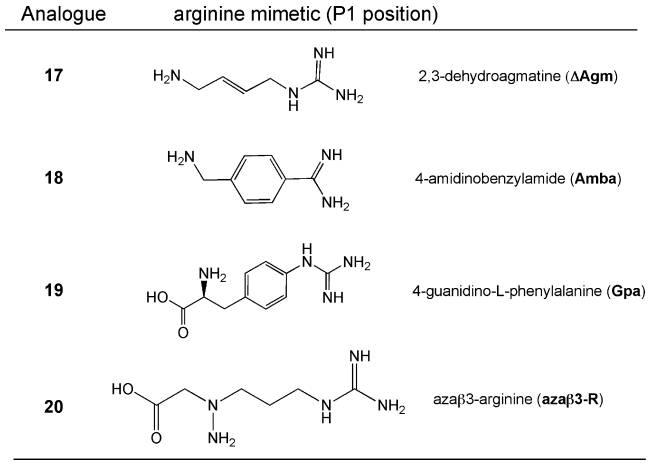

Figure 2. Structure of arginine mimetics used in the present study.

compound with azaβ3-Arg (azaβ3-R) in the P1 position, which is formed by replacing the α-C-atom with a hydrazine moiety. This hydrazinopeptoid was chosen to determine how the loss of chirality and the nitrogen enrichment of the peptide backbone influence the activity of the resulting analogue.

All peptides were obtained via solid-phase peptide synthesis (SPPS) or a combination of SPPS and solution synthesis. The inhibitors containing Arg, Gpa, or the azaβ3-Arg modification at the P1 position (peptides **1**−**16**, **19**, and **20**) and a control-scrambled peptide (Ac-RLRLLKVL-NH_2) were prepared on polystyrene resin (TentaGel S RAM) using standard Fmoc SPPS.

Inhibitors modified with the decarboxylated derivatives ΔAgm or Amba (**17** and **18**) were prepared as described in Scheme 1. First, the P8−P2 segments of these derivatives were synthesized by standard Fmoc SPPS on a 2-chlorotrityl-chloride resin. Next, the protected peptides were cleaved from the resin followed by coupling of Amba·2HCl or ΔAgm$(Boc)_2$ in solution. Side-chain deprotection by TFA was performed in the final step.

Fluorescent versions of the selected analogues were prepared by incorporation of a fluorescein isothiocyanate isomer I (FITC) at the N terminus (βAla was used as a spacer).

All inhibitors were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) and obtained as lyophilized TFA salts. The identity and purity of the compounds were determined by HPLC and matrix-assisted laser desorption/ionization (MALDI) or surface-enhanced laser desorption/ionization (SELDI) mass spectrometry (MS) and by high-resolution mass spectrometry (HRMS). The desired products were obtained in good yield. Information regarding

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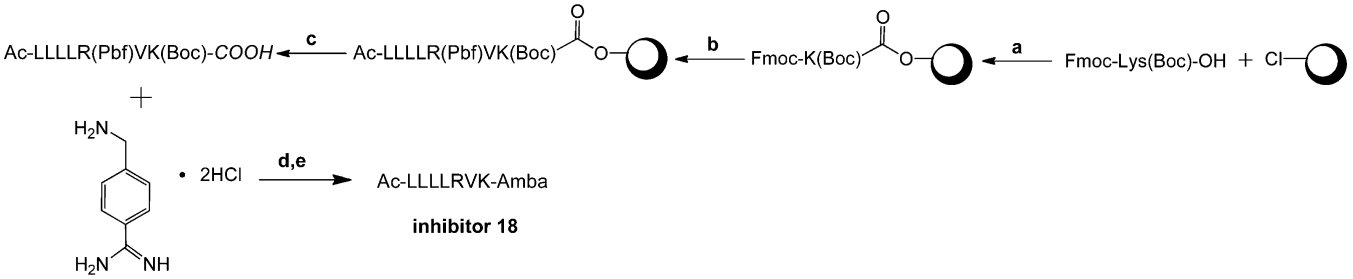
Scheme 1. Synthesis of Inhibitors 17, 18[a]

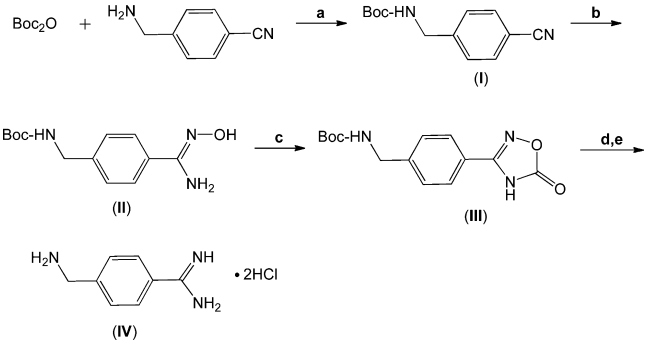


[a]Using the example of the peptide **18**. Reagents and conditions: (a) 2-chlorotrityl chloride resin, 1.2 equiv of Fmoc-Lys(Boc)-OH, 4 equiv of DIPEA, dry DCM, 2.5 h; (b) Fmoc SPPS, coupling with 3 equiv of amino acid, 6Cl-HOBt, and HATU or PyBOP, respectively, and 9 equiv of NMM or DIPEA; (c) HFIP/DCM (1:4, v/v), 2 h; (d) 2 equiv of Amba·2HCl, 2 equiv of COMU, and 4 equiv of NMM, DMF, 12 h; (e) TFA/TIS/H_2O (95:2.5:2.5, v/v/v), 3 h.

the analytical characterization of the inhibitors is provided in the Supporting Information (Tables S1 and S2).

All noncommercially available derivatives (Amba·2HCl, ΔAgm(Boc)$_2$, and Fmoc-azaβ3-Arg(Boc)$_2$) used in the present study were synthesized according to the literature and/or methods developed in our laboratory. Amba·2HCl was prepared as described[24,25] with a slight modification of the acylation step (Scheme 2). To obtain ΔAgm(Boc)$_2$, a novel

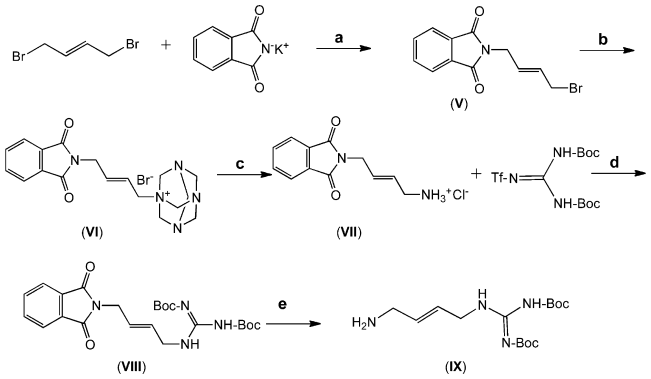
Scheme 2. Synthesis of Amba·2HCl (4-Amidinobenzylamine·2HCl; IV)[a]



[a]Reagents and conditions: (a) H_2O, 1 N NaOH, overnight; (b) NH_2-OH·HCl, DIPEA, MeOH, reflux 12 h; (c) bis(trichloromethyl) carbonate, pyridine/THF, 0 °C, 1 h, 60 °C, 12 h; (d) 50% AcOH(aq), H_2 (50 psi), Pd/C (10%), 2 h; (e) H_2O, HCl, 1.5 h.

synthetic strategy was developed (Scheme 3) on the basis of work by Jeon et al.[26] Briefly, the desired protected ΔAgm compound was obtained in four steps starting from 1,4-dibromo-trans-2-butene. First, the mono-*N*-phthalimide derivative was prepared (**V**). Then, the remaining bromine was converted with hexamethylenetetramine to a quaternary ammonium salt (**VI**), which was hydrolyzed under acidic conditions to a primary amine salt (**VII**). In the final steps, the guanidine fragment was introduced into the molecule (**VIII**) using *N*,*N*′-di-Boc-*N*″-triflylguanidine as the guanidylation reagent followed by the deprotection of the phthalimide group with hydrazine hydrate to give the desired compound, ΔAgm(Boc)$_2$ (**IX**). Full experimental details and analytical data for the compounds **V**–**IX** (including copies of the ^{1}H NMR spectra) are provided in the Supporting Information. Fmoc-azaβ3-Arg(Boc)$_2$ was obtained following the procedure reported by Busnel et al.[27]

Scheme 3. Synthesis of ΔAgm(Boc)$_2$ (*N*-(2-butenyl) guanidine-(Boc)$_2$; IX)[a]



[a]Reagents and conditions: (a) DMF, 48 h; (b) hexamethylenetetramine, $CHCl_3$, 48 h; (c) EtOH, HCl, reflux 2 h; (d) Et_3N, DCM, overnight; (e) MeOH, $CHCl_3$, hydrazine, 4 h.

Kinetic Measurements. Our prior studies that led to the ML-peptide inhibitor development established that the leucine core is essential for its specificity toward PACE4 over furin.[23] To assess the impact of the proposed modifications on the specificity and potency of our leading compound, the inhibition constants (K_i) of the newly synthesized compounds were determined via competitive kinetic assays using recombinant human furin and PACE4. For an analogue modified with Amba, the values of the apparent K_i were calculated according to the equation for the kinetics of reversible tight-binding inhibitors.[28,29] The K_i values of all of the novel compounds together with those of the ML peptide are presented in Figures 3 and 4.

Analogues Modified with Norleucine (Nle). The substitution of Leu by Nle at the P8, P7, and P5 positions had little effect on the inhibitory potency of the resulting analogues (peptides **1**, **2**, and **4**, respectively) against PACE4 (Figure 3A). However, a single modification of the P6 position with Nle yielded compound **3**, which had substantially reduced activity (19-fold less potent than the ML peptide). In the case of peptides with multiple Nle substitutions, all analogues modified at the P6 position (**5**, **8**, and **9**) showed significantly reduced inhibitory activity (8.5–25-fold reduced activity). In contrast, peptides that were not modified at this position (**6**, **7**, and **10**) remained potent PACE4 inhibitors. One of the novel compounds, analogue **10** (Ac-[Nle]LL[Nle]RVKR-NH_2), was even more potent than the ML peptide (K_i = 14 ± 2 vs 20 ± 2 nM for the ML-peptide). Regarding the inhibitory potency

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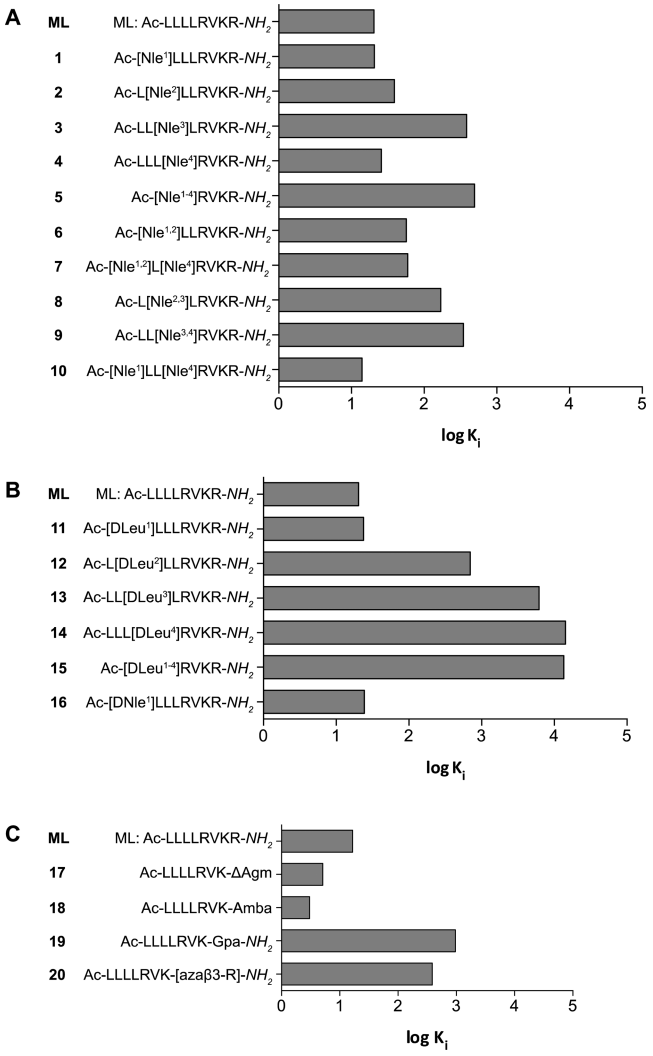

Figure 3. Inhibition constant (K_i) against PACE4 for analogues modified at Multi-Leu core with (A) L-norleucine (Nle), (B) D-amino acids, or (C) arginine mimetics. The results are presented on a logarithmic scale to facilitate comparison. The data for the ML-peptide inhibitor was included as a control and is identical to a previous publication.[23]

toward furin, all analogues with single or multiple Nle substitutions were less potent than the original ML peptide (Figure 4A).

Inhibitors **1** and **10** exhibited significantly improved selectivity toward PACE4 when compared with the ML peptide (31- or 48-fold improvement, respectively, Figure 4A). The selectivity of the two other peptides, analogues **2** and **4**, was slightly improved or maintained. In contrast, all of the newly prepared compounds with Nle at position P6 or with multiple Nle substitutions were poor PACE4 inhibitors with reduced selectivity, with the exception of above-mentioned peptide **10**.

Analogues Modified with D-Leucine (DLeu) and D-Norleucine (DNle). The introduction of the DLeu residue in the structure of the ML-peptide inhibitor yielded analogues with reduced (peptide **12**; 7-fold reduction) or drastically diminished (compounds **13**, **14**, and **15**; 300−700-fold reduction) inhibitory potency toward PACE4. However, peptide **11** (obtained by DLeu substitution at the P8 position) appears to be an exception to the rule because it remained a potent PACE4 inhibitor (K_i = 24 ± 1 nM). On the basis of these results, we designed a single analogue modified with D-norleucine (peptide **16**), whose activity was similar to that of its DLeu counterpart (Figure 3B). Three of the novel peptides (**11**, **13**, and **16**) modified at the P8 or P6 position were 1.7−2.3-fold less effective against furin than the ML peptide (Figure 4B). The inhibitory potency of compounds with DLeu at position P5 or with the entire leucine core with an inverted configuration (Ac-LLL[DLeu]RVKR-NH_2 and Ac-[$DLeu^{1-4}$]-RVKR-NH_2) was strongly decreased (17−22-fold reduction). Moreover, analogue **12**, which was modified at position P7, was 6.6-fold less potent toward furin when compared with the ML-peptide inhibitor. The inhibitory potency of two analogues from this group modified at position P8 (**11** and **16**) against PACE4 was comparable to that of our leading compound; however, their selectivity was greatly improved (≤32-fold, Figure 4B).

Analogues Substituted at Position P1. The incorporation of the decarboxylated and conformationally restricted arginine mimetics ΔAgm and Amba in the structure of the ML peptide (**17** and **18**) yielded analogues with improved inhibitory potency toward PACE4 (K_i = 5.3 ± 1.5 and 3.1 ± 0.8 nM, respectively). In contrast, as shown in Figure 3C, the analogue containing an aromatic amino acid derivative (**19**) had dramatically reduced potency (48-fold reduction). Modification of the structure with an azaβ3-Arg substitution had no beneficial effect on PACE4 inhibition (20-fold diminished activity). Analogues **17** and **18** were also strong furin inhibitors; therefore, these modifications greatly reduced the specificity toward PACE4. For example, the incorporation of Amba drastically improved (100-fold) activity toward furin, which resulted in comparable K_i values for PACE4 and furin (K_i = 3.1 ± 0.8 and 4.3 ± 0.8 nM, respectively). The ΔAgm-substituted inhibitor (**17**) binds to furin with a K_i value of 27 ± 2 nM, resulting in 4-fold diminished specificity toward PACE4 when compared with the ML peptide. The other P1-modified analogues (**19** and **20**) were significantly less potent furin inhibitors, with K_i values of 11.0 and 1.12 μM, respectively.

Although the substrate specificities of PCs differ, they all possess a strong preference for similar multibasic cleavage sequences. Therefore, we selected a few potent PACE4 inhibitors (peptides **1**, **4**, **10**, **11**, and **16**−**18**) and measured their selectivity toward related PCs, hPC2 and hPC7 (Table S2, Supporting Information). The inhibitory activity of the novel compounds against PC7 is similar to that of our leading compound, with the exception of decarboxylated peptides **17** and **18**, which exhibited higher potency (K_i = 72 ± 2 and 13.8 ± 0.4 nM, respectively). In the case of PC2, analogues with substitutions in the multileucine core (**1**, **4**, **10**, **11**, and **16**) were stronger inhibitors (1.7−4-fold increased inhibition) when compared to the ML peptide. The incorporation of the ΔAgm and Amba modifications also significantly increased the inhibitory potency of the resulting analogues toward PC2 (K_i = 1.7 ± 0.6 and 0.24 ± 0.03 nM, respectively).

In Vitro Plasma Stability Studies. Multibasic peptides are potential substrates for a wide variety of trypsin-like proteases that are present in blood; therefore, the plasma stability of our ML-peptide inhibitor and its analogues was a significant parameter to evaluate. We determined the stability profiles of the selected analogues in mouse plasma using RP-HPLC analyses and calculated their half-life ($t_{1/2}$) values. Furthermore, we investigated the degradation pattern of these inhibitors via MS analyses (MALDI-TOF MS). The results of these studies are presented in Figure 5.

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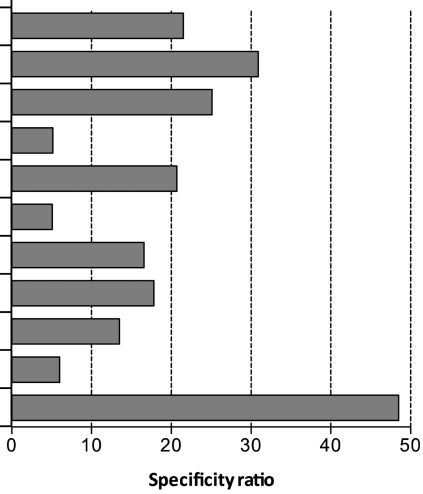
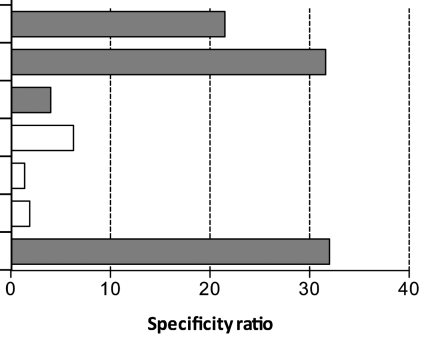
A

	Peptide	K_i (nM) ± SD	
		PACE4	FURIN
ML	ML: Ac-LLLLRVKR-NH_2	20 ± 2	430 ± 10
1	Ac-[Nle^{1}]LLLRVKR-NH_2	21 ± 3	650 ± 10
2	Ac-L[Nle^{2}]LLRVKR-NH_2	39 ± 5	980 ± 300
3	Ac-LL[Nle^{3}]LRVKR-NH_2	385 ± 50	1980 ± 350
4	Ac-LLL[Nle^{4}]RVKR-NH_2	27 ± 8	560 ± 50
5	Ac-[Nle$^{1-4}$]RVKR-NH_2	500 ± 70	2550 ± 200
6	Ac-[Nle^{1,2}]LLRVKR-NH_2	57 ± 5	950 ± 20
7	Ac-[Nle^{1,2}]L[Nle^{4}]RVKR-NH_2	60 ± 10	1070 ± 50
8	Ac-L[Nle^{2,3}]LRVKR-NH_2	170 ± 35	2300 ± 20
9	Ac-LL[Nle^{3,4}]RVKR-NH_2	350 ± 65	2100 ± 100
10	Ac-[Nle^{1}]LL[Nle^{4}]RVKR-NH_2	14 ± 2	680 ± 10



B

	Peptide	K_i (nM) ± SD	
		PACE4	FURIN
ML	ML: Ac-LLLLRVKR-NH_2	20 ± 2	430 ± 10
11	Ac-[DLeu^{1}]LLLRVKR-NH_2	24 ± 1	760 ± 20
12	Ac-L[DLeu^{2}]LLRVKR-NH_2	700 ± 170	2800 ± 200
13	Ac-LL[DLeu^{3}]LRVKR-NH_2	6260 ± 1500	980 ± 30
14	Ac-LLL[DLeu^{4}]RVKR-NH_2	14000 ± 2700	9550 ± 900
15	Ac-[DLeu$^{1-4}$]RVKR-NH_2	14000 ± 3500	7300 ± 1900
16	Ac-[DNle^{1}]LLLRVKR-NH_2	25 ± 4	800 ± 10



C

	Peptide	K_i (nM) ± SD	
		PACE4	FURIN
ML	ML: Ac-LLLLRVKR-NH_2	20 ± 2	430 ± 10
17	Ac-LLLLRVK-ΔAgm	5.3 ± 1.5	27 ± 2
18	Ac-LLLLRVK-Amba	3.1 ± 0.8	4.3 ± 0.8
19	Ac-LLLLRVK-Gpa-NH_2	960 ± 40	11000 ± 1200
20	Ac-LLLLRVK-[azaβ3-R]-NH_2	400 ± 50	1120 ± 100

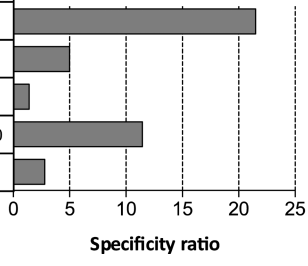


Figure 4. Specificity ratio for PACE4/furin of Multi-Leu analogues modified at the Multi-Leu core with (A) L-norleucine (Nle), (B) D amino acids, and (C) arginine mimetics at the P1 position. Peptides **13**, **14**, and **15** were more specific toward furin. The data for the ML-peptide inhibitor was included as a control and is identical to a previous publication.[23]

The half-life of the ML-peptide inhibitor was 5.1 h. No benefits were observed after the incorporation of norleucines in its structure, and the plasma stabilities of the resulting analogues were reduced in some cases (Figure 5A). Analogues containing D amino acids (DLeu and DNle) had greatly improved proteolytic resistance, with $t_{1/2}$ values of 8.6 and 7.1 h, respectively. Among inhibitors modified at the P1 position, the compound substituted with Amba was 2.4-fold more stable ($t_{1/2}$ = 12 h) than the ML peptide. In contrast, ΔAgm and azaβ3-Arg did not have such a strong impact on the plasma stability, and the half-life of the resulting analogues was 7.2 and 5.6 h, respectively. The first degradation products of the ML inhibitor were detectable after 30 min of incubation at 37 °C in murine plasma, having molecular ions of m/z 897, 769, and 670 (Figure 5C). These molecular ions correspond to seven-, six-, and five-amino acid sequences that lack the C-terminal amino acids (-R-NH_2, -KR-NH_2, and -VKR-NH_2, respectively). As shown in Figure 5C, all of the selected peptides having Arg at the P1 position also followed the same degradation pattern and were cleaved at those specific positions (after basic amino acid residues). The incorporation of decarboxylated arginine mimetics (Amba and ΔAgm) prevented C-terminal degradation after the P3 and P2 positions. However, for all peptides, a

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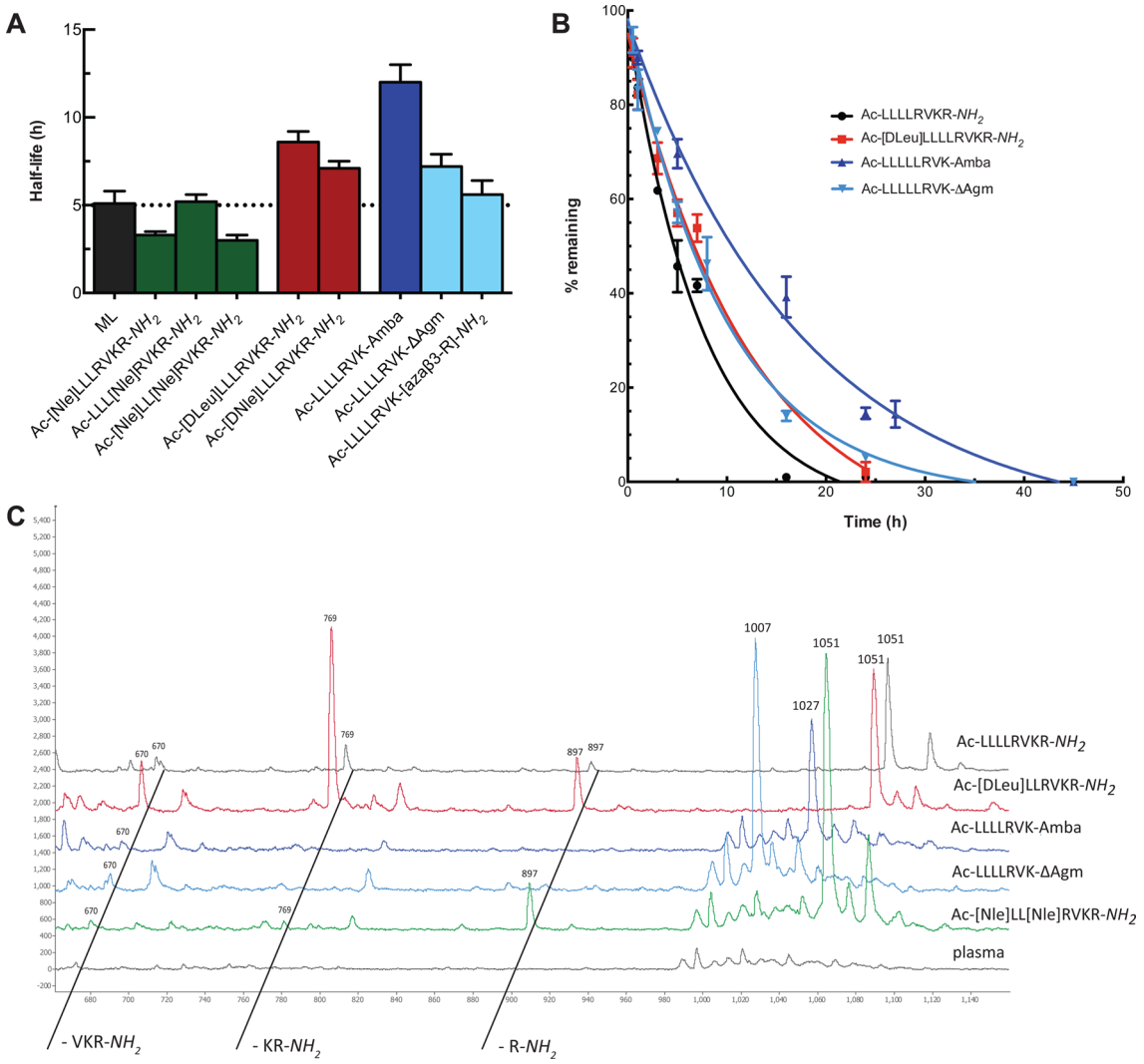

Figure 5. Stability profile of the selected Multi-Leu analogues. The peptides were incubated with the plasma at 37 °C for different time periods. (A) Plasma half-life ($t_{1/2}$) of the ML analogues. (B) Percentage of the peptide remaining at each degradation time point. (C) C-terminal degradation pattern.

molecular ion corresponding to a fragment cleaved after Arg at position P4 was detected.

Antiproliferative Activity and Cytotoxicity against PCa Cell Lines. We previously observed a strong correlation between cellular PACE4 levels and ML-peptide activity in three human PCa cell lines (LNCaP, DU145, and PC3) with different PACE4 expression levels.[23] The ML peptide significantly inhibited the proliferation of DU145 and LNCaP cells (IC_{50} values of 100 ± 10 and 180 ± 60 μM, respectively) and had little effect on PC3 cells, which were used as a negative control because PACE4 is almost undetectable in this cell line. To confirm the specific activity of the ML peptide, we used a scrambled version of this inhibitor (the same amino acid composition but in a different order, a sequence without a PC recognition motif) with the following structure Ac-RLRLLKVL-NH_2. A scrambled peptide had no inhibitory activity against either PACE4 or furin and did not possess antiproliferative properties (Figure S1, Supporting Information).

To determine whether the inhibitory activity of the new ML-peptide analogues against recombinant enzyme could be translated to an antiproliferative effect, we selected several analogues with different inhibitory profiles to perform MTT-based cell proliferation assays. PACE4 inhibitors that were potent in the enzymatic assay exhibited comparable (**1** and **4**) or improved (peptides **10**, **11**, and **16**–**18**) antiproliferative activities relative to the ML-peptide inhibitor (Figure 6A). Among all of the analogues tested, two peptides, **10** and **18**, showed the highest activity against both DU145 (IC_{50} = 40 ± 10 and 25 ± 10 μM, respectively) and LNCaP (IC_{50} = 90 ± 30 and 40 ± 15 μM, respectively) cells (Figure 6B). The antiproliferative activity of analogue **18** substituted with Amba increased 4- and 4.5-fold with DU145 and LNCaP cells, respectively, in comparison to the ML-peptide inhibitor. In contrast, the analogues modified with D amino acids (peptides **11** and **16**) were only slightly more potent than our control peptide (Figure 6A,B) and also displayed cytotoxic activity toward PC3 cells (IC_{50} = 190 ± 50 and 150 ± 20 μM, respectively). As expected, the analogues with poor inhibitory potency toward PACE4 (peptides **3**, **13**, **15**, and **20**) also exhibited weak antiproliferative activity. The relatively high IC_{50} values of peptides **3** and **13** (in DU145 or DU145 and LNCaP cells, respectively) are most likely linked to the nonspecific cytotoxicity of these compounds. Lactate dehydrogenase (LDH) assays were performed with DU145 cells to determine

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A

	Analogue										IC_{50} (μM) ± SEM			LDH (100μM)
											DU145	LNCaP	PC3	DU145
ML	Ac-	L	L	L	L	R	V	K	R	NH_2	100 ± 10	180 ± 60	N.C.	<5%
1	Ac-	[Nle]	L	L	L	R	V	K	R	NH_2	150 ± 40	180 ± 40	N.C.	<5%
3	Ac-	L	L	[Nle]	L	R	V	K	R	NH_2	190 ± 30	N.C	N.C.	<5%
4	Ac-	L	L	L	[Nle]	R	V	K	R	NH_2	110 ± 30	220 ± 40	N.C.	<5%
10	Ac-	[Nle]	L	L	[Nle]	R	V	K	R	NH_2	40 ± 10	90 ± 30	N.C.	<5%
11	Ac-	[Dleu]	L	L	L	R	V	K	R	NH_2	70 ± 30	150 ± 40	190 ± 50	<5%
13	Ac-	L	L	[Dleu]	L	R	V	K	R	NH_2	190 ± 40	130 ± 20	N.C.	<5%
15	Ac-	[Dleu]	[Dleu]	[Dleu]	[Dleu]	R	V	K	R	NH_2	N.C.	N.C.	N.C.	<5%
16	Ac-	[DNle]	L	L	L	R	V	K	R	NH_2	80 ± 10	90 ± 10	150 ± 20	<5%
17	Ac-	L	L	L	L	R	V	K	ΔAgm		90 ± 30	70 ± 20	200 ± 30	<5%
18	Ac-	L	L	L	L	R	V	K	Amba		25 ± 10	40 ± 15	150 ± 35	<5%
20	Ac-	L	L	L	L	R	V	K	azaβ3-R	NH_2	270 ± 10	240 ± 10	N.C	<5%

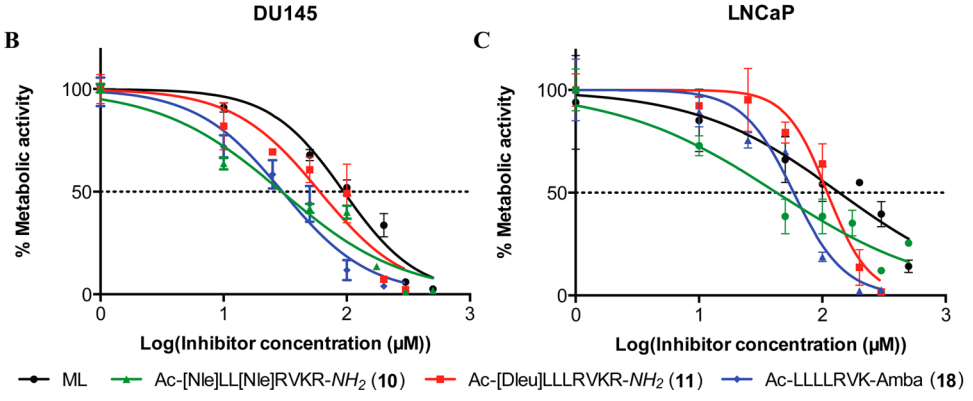

Figure 6. Antiproliferative activity and cytotoxicity of the selected Multi-Leu inhibitors on prostate cancer cell lines. (A) Comparison of the IC_{50} values and lactate dehydrogenase (LDH) levels. The concentration of peptides that inhibited cell growth by 50% (IC_{50}) was determined by MTT cell survival assays on DU145, LNCaP, and PC3 cell lines. N.C. indicates that the curve did not converged to 50% with doses up to 350 μM. Acute cytotoxicity of the analogue at the indicated concentration (100 μM) was measured by lactate dehydrogenase (LDH) assay after 4 h of incubation with DU145 cells. (B, C) Dose−response curves for the antiproliferative effect of the ML-peptide inhibitor and its most potent analogues (peptides **10**, **11**, and **18**) in DU145 and LNCaP cells.

whether the effective concentration of ML-peptide analogues for inhibiting cell proliferation indicates growth inhibition rather than acute cytotoxicity. The activity of LDH, which is an enzyme released into the culture medium from cells with damaged membranes, was determined as an indicator of cell death/lysis severity. After 4 h of incubation with 100 μM of any of the inhibitors, the LDH measurements indicated <5% acute toxicity in DU145 cells (Figure 6A).

Cell Penetration Studies. We previously suggested that the ML-peptide inhibitor targets intracellular PACE4 to inhibit DU145 proliferation, and its N-terminal modification via incorporation of the PEG8 moiety altered this activity by reducing cell permeability.[23] To investigate if the cell penetration efficiency of the ML peptide would be affected by the proposed modifications, the cellular uptake of selected compounds **11** and **17**, which possess the most suitable overall profile (high inhibitory potency in both enzymatic and cell-based assays and improved plasma stability), were compared. This test was performed with FITC-labeled versions of these peptides (FITC-βAla-[DLeu]LLLRVKR-NH_2 and FITC-βAla-LLLLRVK-Amba) after 1 h of incubation with DU145 cells and was analyzed using fluorescence-activated cell sorting (FACS) analyses as previously described.[23] False-positive signals resulting from nonspecific interactions of peptides with the cell membrane were eliminated after trypsinization. The results of these assays are presented in Figure 7 together with the spectra for the ML-peptide inhibitor. In general, the fluorescent version of the ML peptide yielded the highest geometric mean fluorescence intensity (GMFI = 63.92), and the incorporation

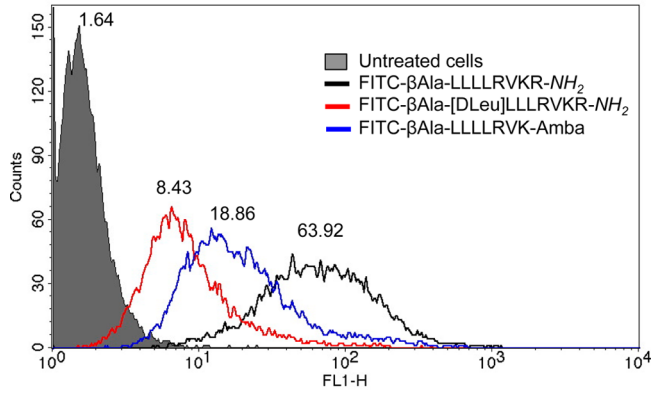

Figure 7. Cellular uptake of the fluorescent ML analogues by DU145 cells. The cells were treated with 10 μM FITC-labeled compounds, and the spectra were collected after 1 h incubation. Untreated cells were used as a control, and their autofluorescence intensity is presented as a gray curve. Numbers indicate the geometric mean fluorescence intensity for peptide-treated cells and autofluorescence intensity of the cells.

of DLeu or Amba into its structure resulted in decreased fluorescence intensities (GMFI = 8.43 and 18.86, respectively), revealing a lower accumulation of those analogues in DU145 cells after 1 h.

DISCUSSION

We previously demonstrated that PACE4 plays a critical role in PCa tumor progression.[21,22] Therefore, the inhibition of this

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protease may offer novel and more effective treatment strategies. These encouraging results prompted us to generate a potent and specific PACE4 inhibitor, which led to the development of the ML peptide.[23] The interesting pharmacological profile of this inhibitor (i.e., specificity toward PACE4, potent antiproliferative effects on PCa cell lines, and good cell permeability) makes the ML peptide an attractive candidate for development as a novel anticancer agent. However, because this molecule is a peptide, the ML-peptide inhibitor encounters additional challenges in the drug-development process, including poor metabolic stability and low bioavailability, which are mainly the result of rapid in vivo degradation by proteases. Therefore, the main goal of this study was to improve the pharmacological profile of the ML peptide through modifications of its structure using a peptidomimetic approach.

The first group of compounds was designed with single or multiple Nle substitutions in the tetraleucine tail of the ML inhibitor. The enzyme kinetic analyses results of these analogues (Figure 4A) demonstrated that the Leu residue in the P6 position plays a significant role in the inhibitory activity against PACE4, and its substitution by the Nle residue resulted in analogues with substantially reduced activity (8.5−19-fold reduction). Interestingly, this relatively small modification of the side chain (replacing the branched alkyl side chain of Leu with the linear side chain of Nle) can cause relatively large changes in the K_i value. In contrast, Nle substitutions at the other positions (alone or in combination) had little effect (peptides **1**, **4**, and **10**) or slightly diminished (compounds **2**, **6**, and **7**) the inhibitory potency toward PACE4. Moreover, two analogues, Ac-[Nle]LLLRVKR-NH_2 (**1**) and Ac-[Nle]LL[Nle]-RVKR-NH_2 (**10**), exhibited improved selectivity profiles when compared with the ML peptide. In the case of peptide **10**, the selectivity toward PACE4 was enhanced nearly 48-fold. Unfortunately, the Nle modifications, despite their beneficial effect on the specificity of the resulting analogues, did not increase their stability in plasma (Figure 5A). However, the potent in vitro activities of the novel Nle-modified inhibitors (analogues **1**, **4**, and **10**) were translated into antiproliferative effects on PCa cells, whereas poor PACE4 inhibitors (peptides **3** and **5**) had significantly reduced efficacy in the cellular assays.

The next group of compounds studied consisted of ML-peptide analogues modified at the tetraleucine region with DLeu or DNle residues (peptides **11**−**16**). The incorporation of D amino acids into biologically active peptides is a well-known strategy to improve their resistance to enzymatic degradation.[30] For PC inhibition, inversion of the configuration of all of the amino acid residues in a potent furin inhibitor, nona-L-arginine (K_i = 40 nM), resulted in a more potent and stable compound, nona-D-arginine, which inhibited furin with a K_i value of 1.3 nM.[31] In contrast, the replacement of one amino acid by its D isomer at the P4−P1 region of PCs substrate in a series of peptidomimetic furin inhibitors destroyed their activity.[32] The same effects were observed for the ML-peptide inhibitor after incorporation of DArg at the P1 position, and the resulting peptide, Ac-LLLLRVK[DArg]-NH_2, was used as a negative control in our previous study.[23] The results presented here clearly demonstrate that the incorporation of D amino acids at the P5, P6, and P7 positions of the ML peptide yielded analogues with strongly diminished inhibitory activity toward PACE4 (peptides **12**−**15**). This effect was stronger when the DLeu residue was closer to the P4−P1 recognition motif (Figure 4B). Only the replacement of Leu at the P8 position by its D isomer was tolerated and did not alter the inhibitory potency toward PACE4 (compounds **11** and **16**), suggesting that the L configuration of the seven amino acid residues (from the P7 to P1 position) is required for proper interaction with the enzyme catalytic pocket. The presence of a DLeu or DNle residue at the P8 position significantly improved the specificity of the resulting analogues toward PACE4 when compared to the ML inhibitor (Figure 4B). However, the major advantage of these two analogues is their enhanced stability in plasma (Figure 5) and improved antiproliferative activity (Figure 6). Interestingly, we observed a considerable difference between the penetration of the fluorescently labeled version of the analogue **11** and the ML peptide into PCa cells (Figure 7). These findings suggest that the incorporation of the DLeu residue at the N terminus significantly affected the cellular uptake of the resulting peptide.

A third group of inhibitors was prepared by incorporating arginine mimetics in the P1 position (**17**−**20**). Among these analogues, only peptides **17** and **18** (with decarboxylated derivatives) exhibited improved activity in enzyme kinetic studies and bound to PACE4 with inhibition constants of 5.3 ± 1.5 and 3.1 ± 0.8 nM, respectively (Figure 4C). The enhanced binding affinities of these inhibitors were most likely induced by the reduction of the conformational freedom at the P1 position with the ΔAgm or Amba modification when compared with the more flexible arginine side chain of the ML inhibitor. Both the presence of an (E) double bond in the side chain of an agmatine derivative and the aromatic ring of 4-amidinobenzylamide stabilized the conformation of the P1 position of the resulting analogues, which presumably facilitated their interactions with the enzyme's catalytic pocket. Interestingly, the other analogue containing rigid aromatic P1 residue (peptide **19**) exhibited poor inhibitory potency, indicating that not only the flexibility reduction but also the proper length and side-chain orientation of the selected modification affects activity. The incorporation of azaβ3-Arg in inhibitor **20** provided no advantage and resulted in an analogue with significantly reduced (20-fold reduction) potency against PACE4. The compounds modified with the ΔAgm and Amba residues, despite their excellent potency against PACE4, suffered from reduced selectivity. For instance, the incorporation of the ΔAgm residue also greatly improved (16-fold) the inhibitory potency of the resulting analogue toward furin and thereby reduced its specificity for PACE4 (to ∼5-fold, compared to the 20-fold specificity of the ML inhibitor against furin; Figure 4C). In the case of peptide **18**, the specificity was even more strongly diminished, and the inhibition constants toward PACE4 and furin were comparable (K_i = 3.1 ± 0.8 and 4.3 ± 0.8 nM, respectively; Figure 4C). Both characterized analogues also displayed limited selectivity toward related PCs and were strong inhibitors of hPC7 and hPC2, which were not observed in the case of the ML inhibitor and its other analogues (Table S2). A similar inhibition profile toward furin and PACE4 was also observed for a series of peptidomimetic furin inhibitors containing the Amba modification at their C termini; however, for those compounds, hPC7 and hPC2 were less affected than furin and PACE4.[12] Predicting the effects of the reduced selectivity of our novel inhibitors in practical use remains challenging. Considering the redundancy among the PCs and their ability to compensate for each other in vivo, it might be beneficial to inhibit more than one enzyme in some pathophysiological situations. However, our recent studies demonstrated that PACE4 plays a critical role in the prostatic neoplastic process that is not duplicated in vivo by other

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cancer-associated PCs, such as furin or PC7.[22] Therefore, a highly specific inhibitor should be also exceptionally effective in treating PCa.

In addition to an excellent inhibitory potency against PACE4 and reduced specificity, both compounds (**17** and **18**) exhibited improved stability profile in plasma (Figure 5). It should be noted that the peptide modified with Amba (**18**) exhibited the highest stability of all of the analogues tested, with $t_{1/2}$ = 12 h. Furthermore, the incorporation of the ΔAgm or Amba modification led to a significant improvement in the antiproliferative activity of the resulting analogues against PCa cell lines (Figure 6). Therefore, both compounds might represent a good starting point for developing a new generation of antiprostate cancer agents. However, bearing in mind that high target selectivity is a primary objective in the drug development process, we are undertaking further SAR studies on our Amba- and ΔAgm-substituted inhibitors to improve their selectivities.

It is interesting to note that the incorporation of the non-natural residue, the Amba modification, also reduced the cell penetration properties of the resulting FITC-labeled peptide, as was seen from a fluorescent version of analogue **11** modified with the DLeu residue (Figure 7).

In conclusion, structure−activity studies of our recently developed ML-peptide inhibitor resulted in several analogues with an improved overall pharmacological profile. The most potent PACE4 inhibitors were obtained by the incorporation of the Amba or ΔAgm residues at the P1 position. These substitutions had the additional benefit of preventing C-terminal proteolytic degradation. Similarly, the replacement of Leu at the P8 position with a DLeu or DNle benefits the stability of the resulting peptides from N-terminal proteases. Overall, these peptides exhibited excellent antiproliferative activities and improved stability profiles. Additional modifications are being sought to provide a compound with unique anti-PCa properties.

EXPERIMENTAL SECTION

Synthesis. *General Procedures.* All solvents and reagents were obtained from commercial suppliers and were used without further purification. All amino acid derivatives and coupling reagents were purchased from Matrix Innovation, Inc. (Montreal, QC, Canada) and ChemImpex International (Wood Dale, IL, USA). TentaGel S RAM resin and 2-chlorotrityl chloride resin were purchased from Rapp Polymere (Tübingen, Germany) and Matrix Innovation, Inc. (Montreal, QC, Canada), respectively. High-performance liquid chromatography was carried out on an Agilent Technologies 1100 system (analytical and semipreparative) equipped with a diode array detector (λ = 210, 230, and 254 nm). The purity of the peptides was determined with an Agilent Eclipse XDB C18 column (5 μm, 4.6 × 250 mm). Semipreparative HPLC was carried out using a Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 80 Å). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA) and [B] acetonitrile: 0.1% aqueous TFA. All inhibitors were obtained as TFA salts after lyophilization. SELDI-TOF mass spectrometry (ProteinChip, Bio-Rad Laboratories, Inc., Hercules, CA, USA) or MALDI-TOF mass spectrometry and HRMS (TripleTOF 5600, ABSciex; Foster City, CA, USA) were used to confirm the identity of the pure products. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Spectrospin 300 NMR instrument with the chemical shifts reported as δ in ppm. The following abbreviations were used to describe spin multiplicity: s, singlet; d, doublet; t, triplet; q, quintet; dd, doublet of doublets; and m, multiplet.

Synthesis of 4-Amidinobenzylamine·2HCl (Amba·2HCl). This compound was prepared as described in the previously[11,24,25] with a slight modification concerning the acylation step (Scheme 2).

A mixture of Boc-4-cyanobenzyloamine (**I**) (50 mmol, 11.6 g), NH_2-OH·HCl (75 mmol, 5.12 g), and DIPEA (75 mmol, 13.06 mL) in methanol was gently stirred for 10 min and then heated under reflux for 12 h. The mixture was cooled, and the solvent was removed in vacuo. Then, the residue was partitioned between ethyl acetate and water. The organic layer was washed with saturated $NaHCO_3$ and brine and dried over anhydrous $MgSO_4$ before being evaporated under reduced pressure to afford Boc-4-(aminomethyl)-*N*-hydroxybenzimidamide (**II**) as a white solid (11.8 g, 89%).

To the solution of product **II** (40 mmol, 10.6 g) in pyridine/THF (40 mL) at 0 °C was added bis(trichloromethyl) carbonate (14 mmol, 4.14 g). The resulting solution was stirred at 0 °C for 1 h and then at 60 °C for 12 h. The mixture was cooled, and then ethyl acetate and 0.5 M HCl were added. The organic layer was successively washed with 0.5 M HCl and brine. The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated to afford 3-(Boc-4-(aminomethyl)phenyl)-1,2,4-oxadiazol-5(4H)-one (**III**) as a yellow solid (9.3 g, 80%).

Next, product **III** was dissolved in 50% aqueous acetic acid (300 mL) and treated with catalyst (10% Pd/C) under 50 psi hydrogen for 2 h. The catalyst was removed by filtration, and the solvent was removed in vacuo. The remaining Boc-4-amidinobenzylamide·acetate was dissolved in water (200 mL) and treated with concentrated HCl (40 mL). The resulting solution was stirred for 1.5 h at rt, the solvent was removed in vacuo, and the residue was lyophilized to afford 4-amidinobenzylamine·2HCl (**IV**) as a white solid (6.5 g). MS: [M + H]$^+$calcd, 149.09; found, 150.0. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.36 (s, 2 H), 7.60−7.80 (2d, 4 H, aromatic). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ (ppm) 42 (CH_2), 128, 129, 130, 138 aromatics), 166 (amidino group).

Synthesis of ΔAgm(Boc)$_2$. Full experimental details and the characterization data for this compound are presented in the Supporting Information.

Synthesis of Fmoc-azaβ3-Arg(Boc)$_2$. This modification was prepared following a previously described procedure.[27] Briefly, a substituted hydrazone was synthesized by the condensation between the aldehyde (3-(Boc-amino)-1-propanal) and commercially available Fmoc-hydrazine. The hydrazone was then reduced by $NaBH_3CN$ to afford the Fmoc-substituted hydrazine. The -CH_2-COOH moiety was introduced by nucleophilic substitution of benzyl bromoacetate. Cleavage of the Boc protecting group and guanidinylation with Goodman's reagent, $(BocNH)_2NTf$, yielded the protected analogue of arginine [Fmoc-azaβ3-Arg$(Boc)_2$].

Peptide Synthesis. The inhibitors containing Arg, Gpa, or azaβ3-Arg modification at position P1 (peptides **1**−**16**, **19**, and **20**) were prepared on polystyrene resin (TentaGel S RAM; Rapp Polymere, capacity 0.23 mmol/g) on a scale of 100 μmol with a Pioneer Peptide Synthesizer (Applied Biosystems) or manually according to standard coupling procedures and Fmoc/Bu^t strategy with HATU as a coupling reagent.[33] Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gpa-$(Boc)_2$, and Fmoc-azaβ3-Arg$(Boc)_2$ were used for the protection of side-chain functionalities. After final Fmoc deprotection, N-terminal acetylation was carried out in DMF with acetic anhydride (0.5%) and 2,6-lutidine (0.6%). After completion of the synthesis, the protected peptidyl resins were treated with a cocktail of TFA/H_2O/TIS (95:2.5:2.5 v/v/v) at room temperature for 3 h. The solutions of the released peptides were filtered and evaporated in vacuo to a volume of about 1 mL. Then, the peptides were precipitated with diethyl ether to afford crude products.

Analogues modified with Amba or ΔAgm were obtained manually by a combination of solid-phase peptide synthesis and solution synthesis. Fmoc-Lys(Boc)-OH (1.2 equiv) was dissolved in dry DCM (15 mL) and DIPEA (4 equiv) and was immediately added to the 2-chlorotrityl chloride resin (1 g, 1 equiv, resin loading: 0.8 mmol/g). The mixture was shaken for 2.5 h at rt followed by treatment with DCM/MeOH/DIPEA (17:2:1 v/v/v), washed several times with DCM, DMF, and DCM, and dried in vacuo. The loading of the resin

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was ~0.4 mmol/g. The synthesis (which was performed on a scale of 200 μmol for each analogue) proceeded via manual Fmoc SPPS according to standard coupling procedures using 3-fold excess of Fmoc amino acids, HATU, or PyBOP (with 6-Cl-HOBt) as coupling agent and DIPEA or NMM (9 equiv) in DMF. The Fmoc groups were removed by treatment with 20% piperidine in DMF. After final Fmoc deprotection, N-terminal acetylation was performed in acetic anhydride/DIPEA/DCM (15:15:70 v/v/v) for 20 min. The protected peptide was cleaved from resin by HFIP/DCM (1:4 v/v) for 2 h at rt. The solvent was removed in vacuo, and the peptide was lyophilized from 50% *t*-butanol in water. Protected peptide (1 equiv) and Amba·2HCl (2 equiv) or ΔAgm(Boc_2) (2 equiv), COMU (2 equiv), and NMM (4 equiv) were dissolved in DMF and stirred for 12 h at rt. The solvent was removed in vacuo to give a brownish oil. This was dissolved in TFA/TIS/H_2O (95:2.5:2.5 v/v/v), stirred for 3 h at rt, precipitated by cold ether, washed two times with ether, and lyophilized.

The FITC peptides were labeled with fluorescein isothiocyanate isomer I (FITC) through their N terminus via the β-Ala spacer. FITC (1.5 equiv) in pyridine−DCM (1:4 v/v) was added to the resin and allowed to couple overnight. Then, peptidyl resins were treated as their counterparts without the FITC moiety.

All peptides were purified by semipreparative HPLC on a C18 column. The appropriate fractions were pooled and lyophilized. The purity of the peptides was controlled using analytical HPLC (Agilent Technologies 1100 system, Waldbronn, Germany) with an Agilent Elipse XDB C18 column. SELDI-TOF MS or MALDI-TOF MS and high-resolution mass spectrometry (TripleTOF 5600, ABSciex, Foster City, CA) were used to confirm the identity of the pure products. According to HPLC and MS analysis, the purity of the peptides exceeded 95%. Their physicochemical properties are presented in Supporting Information Tables S1 and S2.

Biology. *Enzyme Kinetics.* The human enzymes furin, PC2, PACE4, and PC7 were expressed in Drosophila Schneider 2 cells and purified as previously described.[34] All measurements were performed on a Gemini EM 96-well spectrofluorometer (Molecular Devices; Sunnyvale, CA, USA) (λ_{em}, 370 nm; λ_{ex}, 460 nm; cutoff, 435 nm) with pyroGlu-Arg-Thr-Lys-Arg-AMC as substrate (Bachem, Switzerland) at a fixed concentration (Table S4, Supporting Information) in the presence of various inhibitor concentrations at 37 °C over a period of 1 h. Enzyme inhibition assays for furin were determined in 100 mM Hepes, pH 7.5, containing 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 1.8 mg/mL of BSA. All activity measurements with PACE4 and PC7 were performed in 20 mM Bis-Tris, pH 6.5, containing 1 mM $CaCl_2$ and 1.8 mg/mL of BSA, whereas PC2 assays were performed in the same buffer at pH 5.7. Kinetics assays were analyzed using SoftMaxPro5, and K_i values were determined from IC_{50} using Cheng and Prusoff's equation.[35] For analogues modified with Amba, the values for apparent K_i were calculated according to the Morrisson equation for kinetics of reversible tight-binding inhibitors.[28,29]

In Vitro Plasma Stability Studies. Stability measurements of inhibitors were performed by incubating the compounds (0.5 μg/μL) in CD1 mouse plasma from mixed-sex animals collected with heparin sodium (Innovative Research; Novi, MI, USA) at 37 °C. At each time point, the reaction was stopped by adding 150 μL of 1 M guanidium-HCl solution followed by 300 μL of acetonitrile. Proteins were removed by centrifugation, and supernatants were analyzed by reverse-phase HPLC. Peptides AUC were compared to nonincubated samples, and the percentage remaining was plotted to calculate the half-life from one-phase decay curves. Each experiment was performed at least three times in duplicate for each time point. For MALDI-TOF characterization of peptide fragmentation in murine plasma, 100 μg of peptide was incubated at 37 °C. After each incubation at the indicated time points, 5 μL of 3% TFA was added to 10 μL of plasma to stop the enzymatic reaction. Proteins were precipitated by the addition of 2 volumes of acetonitrile followed by 17 000*g* centrifugation. Supernatants were collected, lyophilized, and resolubilized in water with 0.06% TFA. Samples were desalted and partially purified using C18 ZipTip extraction (Millipore) before MALDI-TOF analysis was done using 1 μL of the desalted sample on the ProteinChip Gold Array (Bio-Rad) with 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA; 10 mg/mL; 30:70 acetonitrile/water 0.06% TFA) upon analysis on a ProteinChip SELDI system (Bio-Rad).

Cell Culture. Human prostate cancer cell lines DU145, PC3, and LNCaP were obtained from ATCC (Manassas, VA, USA) and maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with either 5% fetal bovine serum (FBS; Wisent Bioproducts, St-Bruno, Canada) for DU145 or 10% FBS for PC3 and LNCaP. Cells were grown at 37 °C in a water-saturated atmosphere with 5% CO_2.

MTT Proliferation Assays. DU145, LNCaP, and PC3 prostate cancer cells were treated for 72 h with various concentrations of the selected compounds (peptides **1**, **3**, **4**, **10**, **11**, **13**, **15**−**18**, and **20**) to investigate their growth inhibitory activity against those cells. After 24 h, media was changed, and peptides were added to the cells. Cell viability was then assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, as described previously.[23]

LDH Cytotoxicity Assays. The cytotoxicity of the selected compounds was determined using the LDH Cytotoxicity Detection Kit (Promega; Madison, WI, USA), which quantifies the release of lactate deshydrogenase after membrane disruption by an enzymatic assay. Briefly, 24 h after cell plating, peptides were added, and LDH release was measured after 4 h of incubation.

Cellular Uptake. To evaluate the internalization of FITC-labeled inhibitors, DU145 cells were incubated for 1 h in serum-free RPMI media with 10 μM of FITC-βAla-[DLeu]LLLRVKR-NH_2 and FITC-βAla-LLLLRVK-Amba and collected by centrifugation. Following the incubation, cells were washed twice with trypsin (0.05% v/v) for 5 min at 37 °C to remove nonspecific interactions with the membrane proteins. Then, cells were incubated for 2 min with propidium iodine (10 μg/mL) to exclude cells with altered membrane and were immediately analyzed (10 000 events/sample) by fluorescence activated cell sorting (FACS; Becton Dickinson; Mountain View, CA, USA). GeoMeans were determined using CellQuest Software (Becton Dickinson).

Statistical Analysis. All measurements were made in triplicate, and all values are expressed as mean ± SEM (the antiproliferative activity) and mean ± SD values (the inhibitory activity).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analytical data (HPLC and MS analysis) for all inhibitors, general synthetic procedures and analytical data for compounds **V**−**IX**, information for enzyme kinetics, additional inhibition constants, and MTT assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

6-Cl-HOBt, 1-hydroxy-6-chloro-benzotriazole; Ac, acetyl; ΔAgm, 2,3-dehydroagmatine; Amba, 4-amidinobenzylamide; azaβ3-R, azaβ3-arginine; CMK, chloromethyl ketone; COMU, 1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate; DCM, dichloromethane; dec, decanoyl; DIPEA, *N*,*N*-diisopropylethylamine; FITC, fluorescein isothiocyanate isomer I; Fmoc, 9-fluorenylmethoxycarbonyl; DMF, dimethylformamide; Gpa, 4-guanidino-L-phenylalanine; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HFIP, hexafluoro-2-propanol; HRMS, high-resolution mass spectrometry; IC_{50}, concentration of peptides required to inhibit 50% of the cell growth; K_i, inhibition constant; Nle, norleucine; NMM, *N*-methylmorpholine; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ML, Multi-Leu; PCa, prostate cancer; PCs, proprotein convertases; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; SPPS, solid-phase peptide synthesis; $t_{1/2}$, half-life; TFA, trifluoroacetic acid; TIS, triisopropylsilane

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