Bioorganic & Medicinal Chemistry 18 (2010) 2976–2985

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and structure–activity relationship studies of peptidomimetic PKB/Akt inhibitors: The significance of backbone interactions

Yftah Tal-Gan [a], Noam S. Freeman [a], Shoshana Klein [b], Alexander Levitzki [b], Chaim Gilon [a,*]

[a] Institute of Chemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel
[b] Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

ARTICLE INFO

Article history:
Received 21 December 2009
Revised 12 February 2010
Accepted 18 February 2010
Available online 23 February 2010

Keywords:
PKB/Akt
Peptidomimetics
N-Methylation
Peptoid
Solid phase peptide synthesis

ABSTRACT

Elevated levels of activated Protein Kinase B (PKB/Akt) have been detected in many types of human cancer. In contrast to ATP site inhibitors, substrate-based inhibitors are more likely to be selective because of extensive interactions with the specific substrate binding site. Unfortunately, peptide-based inhibitors lack important pharmacological properties that are required of drug candidates. Chemical modifications of potent peptide inhibitors, such as peptoids and N^{α}-methylated amino acids, may overcome these drawbacks, while maintaining potency. We present a structure-activity relationship study of a potent, peptide-based PKB/Akt inhibitor, PTR6154. The study was designed to evaluate backbone modifications on the inhibitory activity of PTR6154. Two peptidomimetic libraries, peptoid and N^{α}-methylation, based on PTR6154, were synthesized and evaluated for in vitro PKB/Akt inhibition efficiency. All the peptoid analogs reduced potency significantly, as well as most of the members of the *N*-methyl library, suggesting that the backbone conformation and/or hydrogen bond interactions of PTR6154 derivatives are essential for inhibition activity. Two N-terminal members of the *N*-methyl library did not decrease potency and can be used as future drug leads.

1. Introduction

Persistently activated Protein kinase B (PKB/Akt) is a phenomenon associated with many types of human cancer, such as breast, colon, ovary, pancreas, head and neck, and prostate cancer.[1] Inhibition of PKB/Akt is therefore an attractive prospect for targeted cancer therapy. Screening of small molecules as enzyme inhibitors is very common. However, in the case of ATP mimetic kinase inhibitors, the resulting small molecule inhibitors usually exhibit low selectivity toward the desired kinase.[2–6] Peptidic substrate-based inhibitors have a much greater potential to become selective inhibitors, due to comprehensive specific interactions with the protein kinase binding site.[7–9] Recently, a library of peptides derived from a PKB/Akt substrate, the protein Glycogen Synthase Kinase 3 (GSK-3), was developed and the interactions of the peptides with PKB/Akt was studied. The peptide Arg-Pro-Arg-Nva-Tyr-Dap-Hol, (PTR6154), derived from the GSK-3 substrate peptide Arg-Pro-Arg-Thr-Ser-Ser-Phe, was found to be a selective, potent PKB/Akt inhibitor.[10] The non-natural amino acids in PTR6154 were intended also to improve peptide stability in vivo. Nonetheless, there are many drawbacks to the use of linear peptides as drug candidates, including rapid metabolism by proteolysis, poor bioavailability and nonselective receptor binding. Peptidomimetics are designed to retain or enhance the biological effects of natural peptides while, at the same time, overcome their undesirable properties. Many types of local and global modifications have been developed in order to form peptidomimetics with improved pharmacological properties.[11–13] Such modified peptides include peptoid and N^{α}-methylated derivatives.

Peptoids are peptide analogs in which one or more of the amino acid side chain residues has been shifted from the α-carbon to the adjacent nitrogen to form an N^{α}-alkylated glycine derivative (Fig. 1).[14] The N-alkylated Gly residue imparts special conformational properties to the peptide structure due to the loss of stereogenicity and reduction of flexibility compared to the parent peptide.

Abbreviations: The abbreviations for amino acids are according to the IUPAC-IUB Commission of Biochemical Nomenclature, http://www.chem.qmul.ac.uk/iupac/AminoAcid/.ACN, acetonitrile; Boc, *t*-butoxycarbonyl; BTC, bis(trichloromethyl)carbonate; DBU, 1,8-diazabicyclo [5,4,0]undec-7-ene; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESI, electrospray ionization; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, (2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HBTU, (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HOAt, 1-hydroxy-7-aza-benzotriazole; HOBt, 1-hydroxybenzotriazole; HRMS, high resolution mass spectrometry; LC, liquid chromatography; MALDI, matrix assisted laser desorption ionization; MBHA, methylbenzhydrylamine; *o*-NBS, *o*-nitrobenzenesulfonyl; *o*-NBS-Cl, *o*-nitrobenzenesulfonyl chloride; pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RP-HPLC, reverse phase high pressure liquid chromatography; SPPS, solid phase peptide synthesis; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TOF, time of flight.

* Corresponding author. Tel.: +972 26586181; fax: +972 26416358.
E-mail address: gilon@vms.huji.ac.il (C. Gilon).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.02.031

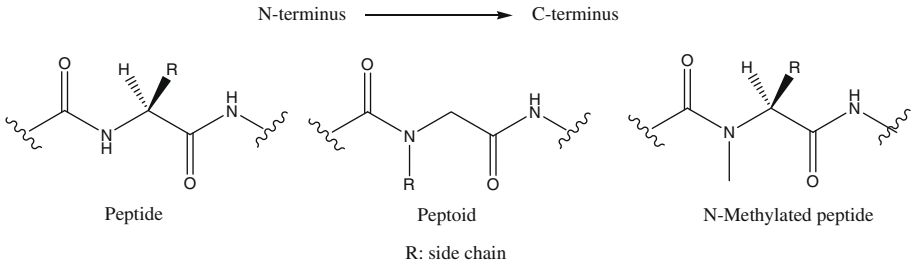


Figure 1. Peptide, peptoid and N-methylated peptide.

The N-alkylated amide bond results in a reduced hydrogen bond donor and increased steric hindrance which may disrupt the classical trans bond configuration. Introducing one peptoid residue may therefore drastically affect peptide conformation and/or binding affinity. Peptoid analogs have shown higher selectivity,[15] improved potency[15] and superior pharmacological properties.[16,17]

N^{α}-Methylation (Fig. 1) is a powerful tool for structure–activity relationship studies and is often used to examine the effects of local backbone modifications on the potency of a known peptide sequence. N-Methylation has been shown to improve important pharmacological parameters such as lipophilicity,[18] bioavailability,[18,19] proteolytic stability,[19,20] conformational rigidity,[21] and duration of action.[19] N-Methylation may also result in enhanced potency,[22–24] new receptor subtype selectivity[25,26] and the conversion of an agonist into an antagonist.[19] N-Methylation induces local backbone constraints caused by steric hindrance,[19,27] it also reduces the number of potential hydrogen bonds formed by the backbone and the tendency to exhibit trans conformations, thereby overall affecting both the secondary and tertiary structures.[28] These effects also influence the ability of the peptide to recognize its binding site, as well as its selectivity and pharmacological properties.[20,24,25,29]

In this report we present structure–activity and structure–stability studies of PTR6154. For the current studies, we designed and synthesized several peptide and peptidomimetic libraries based on PTR6154. We then evaluated the analogs for PKB/Akt inhibition in order to study structure–activity relationships, with emphasis on the effects of backbone modifications on the potency of the inhibitors. We concluded, based on the reduced potency of most of the peptoid and *N*-methyl library members, that the backbone of PTR6154 derivatives has a vital role in potency, affecting conformational flexibility, pharmacophore orientation and/or hydrogen-bond formation. Finally, we compared the lability of the most potent backbone modified inhibitors with that of PTR6154 in the presence of Trypsin/Chymotrypsin. The backbone modified inhibitors that maintained potency were degraded faster than the parent compound, suggesting that a conformational change that favors degradation had occurred.

2. Results and discussion

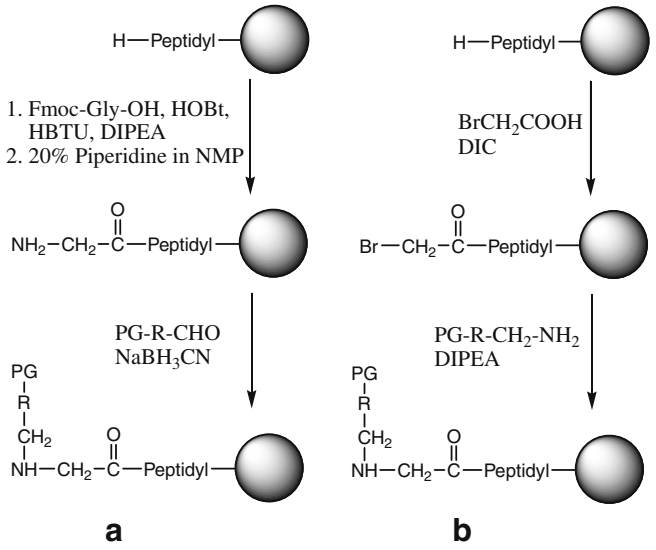
2.1. Chemistry

All the peptides and peptidomimetics were synthesized using standard Fmoc synthesis procedures[30] on Rink amide MBHA resin as the solid support. According to Litman et al.[10] attachment of a cholesteryl moiety enabled the peptide to penetrate into cells, while not impinging upon its potency as a PKB/Akt inhibitor. We attempted to mimic the cholesteryl effect on the N-terminus by preparing both N-terminal free amines and N-terminal acetylated amines. Using a combinatorial approach, we synthesized each compound in one vessel and the resin was divided prior to acetylation in order to provide both analogs.

2.1.1. Synthesis of the peptoid library

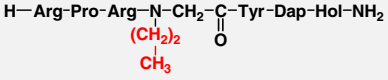
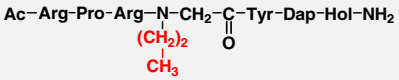
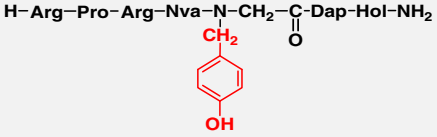
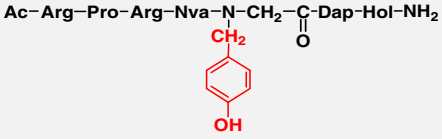
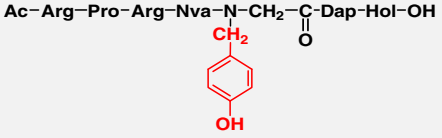
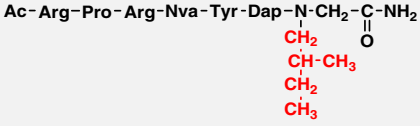
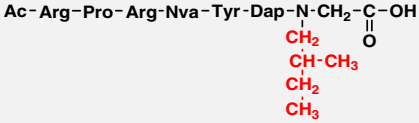
The peptoid derivatives were named according to the modified residue (e.g., peptoid 1 means modification on Arg 1). Two main procedures for solid phase peptoid synthesis have been described. The first is based on the reductive alkylation of glycine with the appropriate aldehyde or ketone to obtain the desired N^{α}-alkylated glycine derivative (Scheme 1a).[31–33] The second method, called the 'sub-monomer' method, is based on the coupling of bromo-acetic acid with the free amine of the growing peptide followed by the introduction of a primary amine bearing the appropriate alkyl side chain residue to obtain the desired N^{α}-alkylated glycine derivative (Scheme 1b).[34] Peptoids 4(a,c), 5(a,c,d) and 7(a,c,d) (Table 1) were synthesized, using readily available aldehydes and amines, by both the methods for comparison. No significant differences were detected between the two methods, either in yield or in product purity. In Peptoid 7(a,c,d), a racemic HoIle peptoid building unit was incorporated instead of a Hol peptoid building unit, for reasons of starting material availability. In order to synthesize Peptoids 1a and 3a (Table 3), we used the microwave assisted reductive alkylation procedure of Park et al.[35]

Coupling of the Fmoc-amino acid to the hindered secondary amine was difficult, but was achieved by Fmoc-amino acid chlorides generated in situ using bis(trichloromethyl)carbonate (BTC) and 2,4,6-collidine in dibromoethane. The couplings were performed at an elevated temperature with longer reaction times[36–38] for Peptoids 4(a,c), 5(a,c,d) and 7(a,c,d). For Peptoids 1a and 3a coupling with the secondary amine was achieved using HATU-mediated coupling procedures.[39]



Scheme 1. Synthetic pathways for N^{α}-alkylated glycine derivative: (a) using reductive alkylation; (b) using the 'sub-monomer' method. R—side chain, PG—protecting group.

Table 1
Inhibitory activity of library 1

Name	Structure	% Inhibition (100 μM)	% Inhibition (5 μM)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	99 ± 1	87 ± 2
YTG 1b	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-OH	95 ± 1	51 ± 3
YTG 1c	Ac-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	92 ± 1	45 ± 2
YTG 1d	Ac-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-OH	66 ± 3	N.D.
Peptoid 4a		16 ± 4	N.D.
Peptoid 4c		0 ± 2	N.D.
Peptoid 5a		85 ± 5	23 ± 4
Peptoid 5c		49 ± 1	N.D.
Peptoid 5d		28 ± 1	N.D.
Peptoid 7a[a]		39 ± 1	N.D.
Peptoid 7c[a]		1 ± 1	N.D.
Peptoid 7d[a]		4 ± 2	N.D.
N-Me 5a	H-Arg-Pro-Arg-Nva-N(Me)Tyr-Dap-Hol-NH_2	88 ± 4	13 ± 1
N-Me 5c	Ac-Arg-Pro-Arg-Nva-N(Me)Tyr-Dap-Hol-NH_2	40 ± 5	N.D.
N-Me 6a	H-Arg-Pro-Arg-Nva-Tyr-N(Me)Dap-Hol-NH_2	75 ± 3	N.D.
N-Me 6b	H-Arg-Pro-Arg-Nva-Tyr-N(Me)Dap-Hol-OH	0 ± 2	N.D.
N-Me 6c	Ac-Arg-Pro-Arg-Nva-Tyr-N(Me)Dap-Hol-NH_2	33 ± 2	N.D.
N-Me 6d	Ac-Arg-Pro-Arg-Nva-Tyr-N(Me)Dap-Hol-OH	1 ± 3	N.D.
N-Me 7a	H-Arg-Pro-Arg-Nva-Tyr-Dap-N(Me)Hol-NH_2	86 ± 3	5 ± 2
N-Me 7c	Ac-Arg-Pro-Arg-Nva-Tyr-Dap-N(Me)Hol-NH_2	29 ± 5	N.D.
N-Me 7d	Ac-Arg-Pro-Arg-Nva-Tyr-Dap-N(Me)Hol-OH	1 ± 4	N.D.

The N-terminal free amine carboxy terminal amide analog is labeled a; the N-terminal free amine carboxy terminal acid analog is b; the N-terminal acetylated carboxy terminal amide analog is c; the N-terminal acetylated carboxy terminal acid analog is d. PKB inhibition was determined according to radioactive kinase assay as described.[2] Inhibition at the concentration of inhibitor indicated in parenthesis is shown as the percent of reduction in PKB activity (0% inhibition = activity in the absence of inhibitor). % Inhibition at 5 μM was determined only for inhibitors that showed over 80% inhibition at 100 μM. N.D. = not determined.

[a] A Holle peptoid building unit was prepared instead of a Hol peptoid building unit.

Cleavage of Peptoids 5c and 7c gave mixtures of two products, 5(c,d) and 7(c,d), that could be easily separated by HPLC. MALDI-TOF MS analysis of the individual products revealed that the MH^+ species of d was 1 mass unit heavier than the MH^+ of c. This phenomenon has been reported earlier for N-alkylated peptide bonds, suggesting the hydrolysis of the carboxy terminal amide into the acid form.[40,41] Negative MALDI-TOF MS as well as Exact Mass analysis supported this hypothesis (Table 5). We observed carboxy terminal hydrolysis of several of the other peptides reported here as well (Tables 6 and 7).

2.1.2. Synthesis of the N^{α}-methylation library

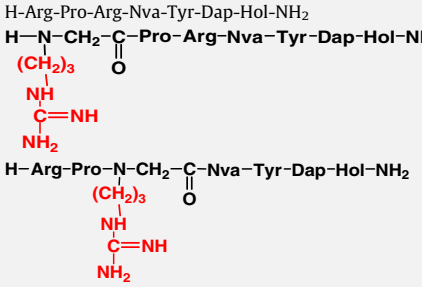
The *N*-methyl derivatives were named according to the modified residue (e.g., N-Me 1 means modification on Arg 1). N^{α}-Methylation was performed according to the procedure of Biron et al.[42] For N-Me 5(a,c), 6(a-d) and 7(a,c and d) coupling to the hindered secondary amine was achieved using BTC as a coupling reagent (Table 1). Coupling to the N^{α}-methylated amino acid in N-Me 2a, 3(a,b) and 4a (Table 3) was not successful using BTC as the coupling reagent, even when the temperature was elevated to 80 °C and the reaction time was extended to 72 h. Repeating the

Table 2
Inhibitory activity of library 2a

Name	Structure	% Inhibition (20 μM)	% Inhibition (5 μM)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	96 ± 1	87 ± 2
YTG 2a	H-Arg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	96 ± 1	88 ± 2
YTG 2c	Ac-Arg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	94 ± 1	83 ± 3
YTG 3a	H-DArg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	40 ± 4	N.D.
YTG 3b	H-DArg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-OH	69 ± 6	N.D.
YTG 4a	H-Lys-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	92 ± 1	73 ± 2
YTG 4c	Ac-Lys-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	85 ± 3	61 ± 4
YTG 4d	Ac-Lys-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-OH	46 ± 1	N.D.
YTG 5a	H-DLys-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	3 ± 2	N.D.
YTG 5b	H-DLys-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-OH	48 ± 4	N.D.

The N-terminal free amine carboxy terminal amide analog is labeled a; the N-terminal free amine carboxy terminal acid analog is b; the N-terminal acetylated carboxy terminal amide analog is c; the N-terminal acetylated carboxy terminal acid analog is d. PKB inhibition was determined according to radioactive kinase assay as described.[2] Inhibition at the concentration of inhibitor indicated in parenthesis is shown as the percent of reduction in PKB activity (0% inhibition = activity in the absence of inhibitor). % Inhibition at 5 μM was determined only for inhibitors that showed over 80% inhibition at 20 μM. N.D. = not determined.

Table 3
Inhibitory activity of library 2b

Name	Structure	% Inhibition (100 μM)	% Inhibition (5 μM)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	99 ± 1	87 ± 2
Peptoid 1a		95 ± 1	32 ± 4
Peptoid 3a		30 ± 4	N.D.
N-Me 1a	H-N(Me)Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	97 ± 2	85 ± 4
N-Me 2a[a]	H-Arg-N(Me)Ala-Arg-Nva-Tyr-Dap-Hol-NH_2	95 ± 3	77 ± 2
N-Me 3a	H-Arg-Pro-N(Me)Arg-Nva-Tyr-Dap-Hol-NH_2	92 ± 1	39 ± 3
N-Me 3b	H-Arg-Pro-N(Me)Arg-Nva-Tyr-Dap-Hol-OH	42 ± 3	N.D.
N-Me 4a	H-Arg-Pro-Arg-N(Me)Nva-Tyr-Dap-Hol-NH_2	62 ± 2	N.D.

The N-terminal free amine carboxy terminal amide analog is labeled a; the N-terminal free amine carboxy terminal acid analog is b; the N-terminal acetylated carboxy terminal amide analog is c; the N-terminal acetylated carboxy terminal acid analog is d. PKB inhibition was determined according to radioactive kinase assay as described.[2] Inhibition at the concentration of inhibitor indicated in parenthesis is shown as the percent of reduction in PKB activity (0% inhibition = activity in the absence of inhibitor). % Inhibition at 5 μM was determined only for inhibitors that showed over 80% inhibition at 100 μM. N.D. = not determined.

[a] N^{α}-Methylated alanine was chosen as the proline derivative.

Table 4
Inhibitory activity of library 3

Name	Structure	% Inhibition (50 μM)	% Inhibition (5 μM)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	98 ± 1	87 ± 2
YTG 6a	H-Arg-Pro-Arg-Val-Tyr-Dap-Phe-NH_2	77 ± 1	N.D.
YTG 6b	H-Arg-Pro-Arg-Val-Tyr-Dap-Phe-OH	12 ± 5	N.D.
YTG 7a	H-Arg-Pro-Arg-Thr-Ser-Dap-Phe-NH_2	22 ± 1	N.D.
YTG 8a	H-Arg-Pro-Arg-Nva-Tyr-DDap-Hol-NH_2	33 ± 1	N.D.
YTG 9a	H-Arg-Pro-Arg-Nva-Tyr-Cys-Hol-NH_2	99 ± 1	95 ± 1

The N-terminal free amine carboxy terminal amide analog is labeled a; the N-terminal free amine carboxy terminal acid analog is b. PKB inhibition was determined according to radioactive kinase assay as described.[2] Inhibition at the concentration of inhibitor indicated in parenthesis is shown as the percent of reduction in PKB activity (0% inhibition = activity in the absence of inhibitor). % Inhibition at 5 μM was determined only for inhibitors that showed over 80% inhibition at 50 μM. N.D. = not determined.

coupling with HATU as the coupling reagent overcame this difficulty and yielded the desired N^{α}-methylated peptides.

C-Terminal deamination was also observed in the *N*-methyl library; cleavage of N-Me 3a, 6(a,c) and 7c gave both the carboxy terminal amide and acid forms (Table 6).

2.2. Biological screening

All peptides and peptidomimetics were screened for inhibition of PKB/Akt in a cell-free radioactive assay, and compared with PTR6154 (Tables 1–4). IC_{50} values (Table 8) were determined for the most potent inhibitors, identified in the initial screening.

2.2.1. The first inhibitor library

Table 1 was designed to evaluate the effect of amino-terminal acetylation on the potency of PKB/Akt inhibition. This library comprised matched amino-terminal acetylated and non-acetylated derivatives of PTR6154, as well as a few peptoids and N^{α}-methylated compounds

Table 5
MS characterization of the peptoid compounds

Name	Calcd MH^+	Observed MH^+ (MALDI)	Calcd $M(-H)^-$	Observed $M(-H)^-$ (MALDI)	Calcd MH_2^{2+}	Observed $MH_2^{2+}/2$ (HRMS)	Calcd MHA^{+a}	Observed MHA^{+a} (HRMS)
Peptoid 1a	902.57	902.43	900.55	—	903.5756	451.7751	1016.5611	1016.5226
Peptoid 3a	902.57	902.67	900.55	—	903.5756	451.7794	1016.5611	1016.5409
Peptoid 4a	902.57	902.49	900.55	—	903.5756	451.7695	1016.5611	1616.5187
Peptoid 4c	944.58	944.51	942.56	—	945.5861	472.7951	1058.5717	1058.5720
Peptoid 5a	902.57	902.29	900.55	—	903.5756	451.7757	1016.5611	1016.5317
Peptoid 5c	944.58	944.23	942.56	—	945.5861	472.7912	1058.5717	1058.5546
Peptoid 5d	945.56	945.22	943.55	943.16[b]	946.5701	473.2842	1059.5557	—
Peptoid 7a	902.57	902.49	900.55	—	903.5756	451.7861	1016.5611	1016.5434
Peptoid 7c	944.58	944.39	942.56	942.26[b]	945.5861	472.7927	1058.5717	1058.5578
Peptoid 7d	945.56	945.27	943.55	943.16	946.5701	473.2834	1059.5557	—

[a] MHA^+ is an adduct with TFA.
[b] Minor peak.

Table 6
MS characterization of the N^{α}-methyl compounds

Name	Calcd MH^+	Observed MH^+ (MALDI)	Calcd $M(-H)^-$	Observed $M(-H)^-$ (MALDI)	Calcd MH_2^{2+}	Observed $MH_2^{2+}/2$ (HRMS)	Calcd MHA+[a]	Observed MHA+[a] (HRMS)
N-Me 1a	916.58	916.65	914.57	—	917.5912	458.7965	1030.5768	1030.5630
N-Me 2a	890.57	890.02	888.55	—	891.5756	445.7878	1004.5611	1004.5424
N-Me 3a	916.58	916.41	914.57	—	917.5912	458.7964	1030.5768	1030.5585
N-Me 3b	917.57	917.24	915.55	915.33	918.5752	459.2876	1031.5608	—
N-Me 4a	916.58	916.36	914.57	—	917.5912	458.7965	1030.5768	1030.5764
N-Me 5a	916.58	916.48	914.57	—	917.5912	458.7966	1030.5768	1030.5635
N-Me 5c	958.59	958.44	956.58	—	959.6018	479.7994	1072.5874	1072.5698
N-Me 6a	916.58	916.34	914.57	914.29[b]	917.5912	458.7937	1030.5768	1030.5534
N-Me 6b	917.57	917.38	915.55	915.43	918.5752	459.2870	1031.5608	—
N-Me 6c	958.59	958.38	956.58	—	959.6018	479.8001	1072.5874	1072.5721
N-Me 6d	959.58	959.45	957.56	957.36	960.5858	480.2914	1073.5714	—
N-Me 7a	916.58	916.50	914.57	—	917.5912	458.7950	1030.5768	1030.5574
N-Me 7c	958.59	958.40	956.58	—	959.6018	479.7988	1072.5874	1072.5650
N-Me 7d	959.58	959.20	957.56	957.11[b]	960.5858	480.2927	1073.5714	—

[a] MHA^+ is an adduct with TFA.
[b] Minor peak.

Table 7
MS characterization of the linear peptides

Name	Calcd MH^+	Observed MH^+ (MALDI)	Calcd $M(-H)^-$	Observed $M(-H)^-$ (MALDI)	Calcd MH_2^{2+}	Observed $MH_2^{2+}/2$ (HRMS)	Calcd MHA+[a]	Observed MHA+[a] (HRMS)
YTG 1b	903.55	903.34	901.54	900.97	904.5596	452.2713	1017.5452	—
YTG 1c	944.58	944.49	942.56	942.37[b]	945.5861	472.7907	1058.5717	1058.5707
YTG 1d	945.56	945.52	943.55	943.42	946.5701	473.2755	1059.5557	—
YTG 2a	1058.67	1058.25	1056.65	—	1059.6767	529.8278	1172.6623	—
YTG 2c	1100.68	1100.47	1098.67	—	1101.6872	551.3337	1214.6728	—
YTG 3a	1058.67	1058.73	1056.65	—	1059.6767	529.8294	1172.6623	—
YTG 3b	1059.65	1059.51	1057.64	1057.28	1060.6607	530.3292	1173.6463	1173.6459
YTG 4a	1030.66	1030.48	1028.65	—	1031.6705	515.8278	1144.6561	1144.6338
YTG 4c	1072.67	1072.56	1070.66	1070.40	1073.6811	536.8338	1186.6667	1186.6460
YTG 4d	1073.66	1073.58	1071.64	1071.30	1074.6651	537.3245	1187.6507	—
YTG 5a	1030.66	1030.55	1028.65	—	1031.6705	515.8251	1144.6561	1144.6275
YTG 5b	1031.65	1031.68	1029.63	1029.43[b]	1032.6545	516.3259	1145.6401	—
YTG 6a	922.54	922.43	920.52	920.26[b]	923.5443	461.7644	1036.5298	1036.5119
YTG 6b	923.52	923.42	921.51	921.05	924.5283	462.2571	1037.5139	—
YTG 7a	848.48	848.32	846.47	—	849.4922	424.7402	962.4778	962.4578
YTG 8a	902.57	902.29	900.55	—	903.5756	451.7809	1016.5611	1016.5440
YTG 9a	919.53	919.42	917.51	—	920.5367	459.7634	1033.5223	1033.0159

[a] MHA^+ is an adduct with TFA.
[b] Minor peak.

2.2.2. The N and C terminal effect

The compounds with acetylated amino termini were much less potent than the corresponding free amine derivatives (e.g., PTR6154 87% inhibition vs YTG 1c 45% at 5 μM; Peptoid 7a 39% inhibition vs 7c 1% at 100 μM; N-Me 5a 88% inhibition vs 5c 40% at 100 μM). There was also a significant decrease in PKB/Akt inhibition when the carboxy terminal acid form of a peptide was compared with the amide form (e.g., PTR6154 87% inhibition vs YTG 1b 51% at 5 μM; Peptoid 5c 49% inhibition vs 5d 28% at 100 μM; N-Me 6a 75% inhibition vs 6b 0% 100 μM).

Table 8
IC_{50} values of the selected compounds

Name	Structure	IC_{50} (μM) (95% confidence)
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	0.94 (0.78–1.14)
N-Me 1a	H-N(Me)Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	0.92 (0.50–1.71)
N-Me 2a	H-Arg-N(Me)Ala-Arg-Nva-Tyr-Dap-Hol-NH_2	2.03 (1.65–2.51)
YTG 2a	H-Arg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	0.84 (0.33–2.11)
YTG 2c	Ac-Arg-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	1.17 (0.46–3.00)
YTG 9a	H-Arg-Pro-Arg-Nva-Tyr-Cys-Hol-NH_2	0.40 (0.35–0.46)

IC_{50} of PKB inhibition was determined according to radioactive kinase assay as described.[2] IC_{50} values and 95% confidence range (parenthesis) were determined using Graphpad Prism 5.

2.2.3. The backbone modification effect

In this initial library, backbone modifications decreased potency, suggesting that the local constraints induced by the modifications, or the inability of the backbone to produce the same number of hydrogen bonds as the parent PTR6154, reduced PKB/Akt inhibition. Peptoid 5a (23% inhibition at 5 μM) showed the highest inhibition efficiency of all the peptoids in this library. N-Me 5a, 6a and 7a (88%, 75% and 86% inhibition at 100 μM accordingly) had similar potencies to one another, as did N-Me 5c, 6c and 7c (40%, 33% and 29% inhibition at 100 μM accordingly), suggesting that although methylation strongly decreased potency, the position of the methylation site (at least with regard to residues 5–7) was less significant. In accordance with these observations, we decided to complete the peptoid and N^{α}-methyl scan without amino-terminal acetylation. Furthermore, we decided to examine more closely the amino-terminal effect by adding N-terminal positively charged D/L amino acids (Arg or Lys) to PTR6154, as spacers, before the acetylation.

2.2.4. The second inhibitor library

Tables 2 and 3 confirmed that a positively charged amino terminus contributes to the potency. Acetylation of the amino terminus after the addition of a highly positively charged Arg spacer did not impair potency (Table 2, compare YTG 2a 88% inhibition and 2c 83% inhibition at 5 μM; and see below). Acetylation after the addition of the less positively charged Lys spacer slightly reduced potency compared with the free amine derivative (Table 2, YTG 4a 73% inhibition versus 4c 61% at 5 μM), but the effect was much smaller than that of acetylation without an additional positively charged N-terminal amino acid side chain (Table 1, PTR6154 87% inhibition versus YTG 1c 45% at 5 μM). This observation implies that intensifying the inhibitory effect depends on the attachment of a positively charged N-terminus to a negatively charged cluster.

2.2.5. The N-terminus D-amino acid effect

Interestingly, the addition of N-terminal D-Arg or D-Lys decreased potency significantly (Table 2, YTG 3a 40% inhibition and 5a 3% at 20 μM, respectively). The carboxy terminal acid form, however, showed higher potency than the amide form for these sequences (Table 2, compare YTG 3a 40% inhibition to 3b 69% at 20 μM, and 5a 3% inhibition to 5b 48% at 20 μM). We presume that the addition of a D-amino acid to the N-terminus leads to a major change in the active conformation.

2.2.6. The peptoid and N-methylation effect

The peptoid and N^{α}-methylation scans performed in libraries 1 and 2 (Tables 1 and 3), showed that, in general, these backbone modifications reduced the potency of the inhibitor. Exceptions to this rule were N-Me 1a and N-Me 2a, which will be incorporated in the possible drug lead compounds (see below).

2.2.7. The third inhibitor library

We next decided to introduce changes in the amino acid sequence of the lead inhibitor, PTR6154 (Table 4). In PTR6154, the three residues surrounding the phosphorylation site in the original substrate peptide were changed, mainly into non-proteinogenic amino acids.[10] Attempts to modify these residues, such as by replacing the non-proteinogenic amino acids Hol and Nva with proteinogenic amino acids (YTG 6(a,b)), or with the residues present in the original substrate (YTG 7a), resulted in reduced potency (77%, 12% and 22% inhibition at 50 μM, respectively). In PTR6154, the Ser residue of the original substrate was replaced by a non-phosphorylatable mimetic, diaminopropionic acid (Dap).[10] Since the Ser-Dap replacement alone did not yield a potent inhibitor (YTG 7a), we investigated the role of the Dap residue. As expected, changing the chirality of the Dap residue strongly impaired potency (YTG 8a 33% inhibition at 50 μM). Interestingly, replacement of the Dap residue with Cys maintained potency (YTG 9a 95% inhibition at 5 μM), suggesting that nucleophilicity of the side chain might be essential for the inhibition.

2.3. IC_{50} values

The most promising inhibitors from the initial screens were: (A) the peptides YTG 2(a,c) in which an additional Arg was added to the N-terminus (Table 2); (B) the backbone modified peptides N-Me 1a and 2a (Table 3); and (C) peptide YTG 9a, in which the Dap residue was replaced by Cys (Table 4). IC_{50} values were determined for these compounds (Fig. 2 and Table 8).

2.3.1. N-Terminal Arg addition

YTG 2(a,c) had very similar IC_{50} values to PTR6154 (0.84, 1.17 and 0.94 μM, respectively), confirming our assumption that the addition of the positively charged Arg residue compensates for the decrease in potency which resulted upon acetylation (YTG 2c).

2.3.2. N-Methylation

N-Me 2a was slightly less potent than PTR6154 (IC_{50} values of 2.03 and 0.94 μM, respectively) and N-Me 1a was equally potent as PTR6154 (IC_{50} values of 0.92 and 0.94 μM, respectively). In N-Me 2a the original residue, prior to methylation, was proline. Proline has a secondary amine and is known to induce local constraints to the peptide sequence.[43] Proline acts as a secondary structure (α-helix, β-sheet) terminator and induces turn motifs (such as β-turns) into the secondary structure. We substituted the proline residue with N(Me)Ala, which also has a secondary amine that cannot act as a hydrogen bond donor. Introducing N(Me)Ala could not induce hydrogen bonding, which may explain why there was no major effect on potency. The slightly lower potency may be attributed to a slightly different kink induced by the more flexible N(Me)Ala compared to the more rigid proline. The comparable potency of N-Me 1a and PTR6154 is quite understandable considering that the N-methylation is on the N-terminus. N-Terminal methylation is flexible and hardly causes any backbone constraint, while the N^{α}-methylated amine can still act as a hydrogen bond donor.

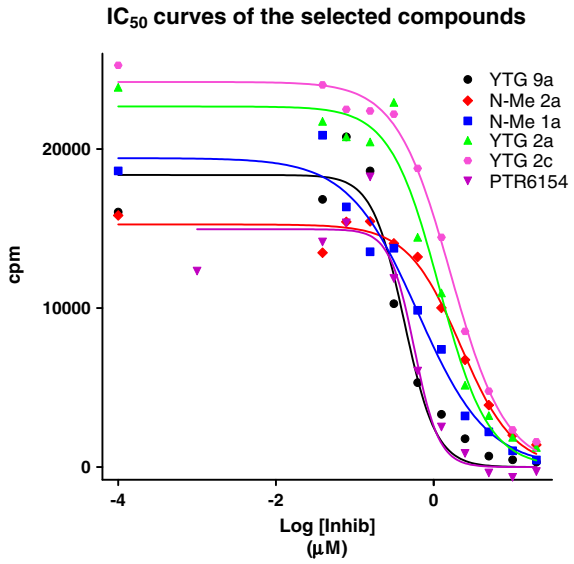


Figure 2. IC_{50} curves of the selected compounds produced using Graphpad Prism 5. Representative curves are shown.

2.3.3. Dap/Cys replacement

The enhanced potency of YTG 9a compared with PTR6154 (IC_{50} values of 0.40 and 0.94 μM, respectively) suggests that Cys might be a better isostere of Ser than Dap. This merits further investigation.

2.4. Stability study

The two backbone modified peptidomimetics, N-Me 1a and 2a, along with PTR6154, were incubated at 37 °C in a mixture of trypsin and chymotrypsin and their degradation was monitored by HPLC and MS (Fig. 3). Interestingly, N-Me 1a and 2a (Fig. 3b and c) were degraded faster than PTR6154 (Fig. 3a). PTR6154 showed ~40% degradation after 30 min, ~70% after 1 h and complete degradation after 2 h. N-Me 1a showed complete degradation in less than 30 min, and N-Me 2a showed ~70% degradation after 30 min, ~90% after 1 h and complete degradation within 90 min. Considering that the N-methylation sites are at the N-terminus and that N(Me)Ala replaced proline, we did not expect these peptides to show higher resistance toward degradation than PTR6154. However, increased degradation was not expected. The cleavage sites were deduced from the MS of the resultant fragments (Table 9). None of the cleavage sites were adjacent to the methylated residues, therefore, these results do not rule out the possibility that N-methylation of cleavage sites can enhance peptide stability. Since the other N-methylated peptides were much less potent than PTR6154, we did not evaluate their resistance to degradation. These results suggest that the conformational changes caused by N-methylation of amide bonds distant from the cleavage site can result in reduced resistance to degradation.

3. Conclusions

We present here a structure–activity relationship study of PTR6154, a substrate-based peptide inhibitor of PKB/Akt.[10] We discovered that a positive charge at the amino terminus is important for inhibitor potency. The positive charge can be contributed by the amino terminus itself, in the form of free amine, or by a positively charged amino acid, preferably Arg. Our results suggest that the extension of the inhibitor sequence by one or several Arg residues should maintain potency while enabling the addition of moieties to enhance permeability, such as cholesteryl.

Most of the backbone modifications introduced led to a dramatic decrease in potency. All of the peptoid analogs had negligible potency, and the 'best' peptoids were still 10-fold less active than PTR6154. Similarly, N^{α}-methylation of the amino acid residues in the inhibitor strongly compromised inhibition, with the exception of the N-terminal amine and N(Me)Ala replacing proline. Our results indicate that the hydrogen bonds formed by the backbone have important roles in site recognition and affinity to the enzyme. These results further suggest that conformational freedom is vital for proper substrate–enzyme complementarity fit and therefore, any local constraints induced by steric effects reduce efficacy significantly.

The most potent backbone modified peptides, N-Me 1a and 2a, were less resistant to degradation than PTR6154, implying that backbone modifications can lead to conformational changes that impair stability and that the modification site is important for obtaining enhanced stability.

We found that replacement of the Dap residue by Cys increased potency, probably by mimicking the electronic charge of Ser better. This alteration is being further investigated to determine whether or not it is a better drug lead than the parent peptide PTR6154.

4. Methods

4.1. Chemistry. General

All starting materials were purchased from commercial sources and were used without further purification. HRMS spectra were recorded on ESI microTOF-LC (Bruker Daltonik GmbH, Bremen). MALDI-TOF spectra were recorded on a PerSeptive Biosystems MALDI-TOF MS, using α-cyano-4-hydroxycinnamic acid as matrix. CEM Discover synthesizer (USA) was used for Microwave assisted SPS.

All analytical HPLC were recorded at 220 nm at a flow rate of 1 ml/min on an RP-18 column (5μ 250 × 4.6 mm, 110 Å), Eluents A (0.05% TFA in TDW) and B (ACN) were used in a linear gradient (95% A→5% A in 35 min). Preparative HPLC was recorded at 220 nm on an RP-18 column (10μ 250 × 10 mm, 110 Å), Eluents A (0.05% TFA in TDW) and B (ACN) were used in a linear gradient (95% A→75% A in 30 min) at a flow of 5 ml/min.

4.2. General methods for SPPS

Swelling: The resin was swelled for at least 2 h in CH_2Cl_2. Fmoc removal: The resin was treated with a solution of 20% piperidine in 1-methyl-2-pyrrolidinone (NMP) (2 × 20 min), then washed with NMP (5 × 2 min). Microwave assisted Fmoc removal: The resin was treated with a solution of 20% piperidine in NMP and the reaction was conducted by microwave irradiation (50 W, 75 °C, 3 min). The procedure was repeated once and the resin was washed with NMP (×3). HBTU coupling: Fmoc protected amino acids (1.5 equiv) were dissolved in NMP. *N*,*N*-Diisopropylethylamine (DIPEA) (1.5 equiv) and 1-hydroxybenzotriazole (HOBt) were added and the mixture was cooled to 0 °C. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.5 equiv) was added and the mixture was pre-activated by mixing for 10 min, added to the resin, and shaken for 1 h. The resin was washed with NMP (3 × 2 min). Microwave assisted HBTU coupling: Fmoc protected amino acids (3 equiv) were dissolved in NMP. DIPEA (3 equiv) and HBTU (3 equiv) were added, the mixture was pre-activated by mixing for 1 min and added to the resin. The reaction was conducted by microwave irradiation (25 W, 75 °C, 5 min) and the resin was washed with NMP (×3). Capping: The resin was treated with a solution of Ac_2O (10 equiv) and DIPEA (7.15 equiv) in dimethylformamide (DMF) for 20 min and washed

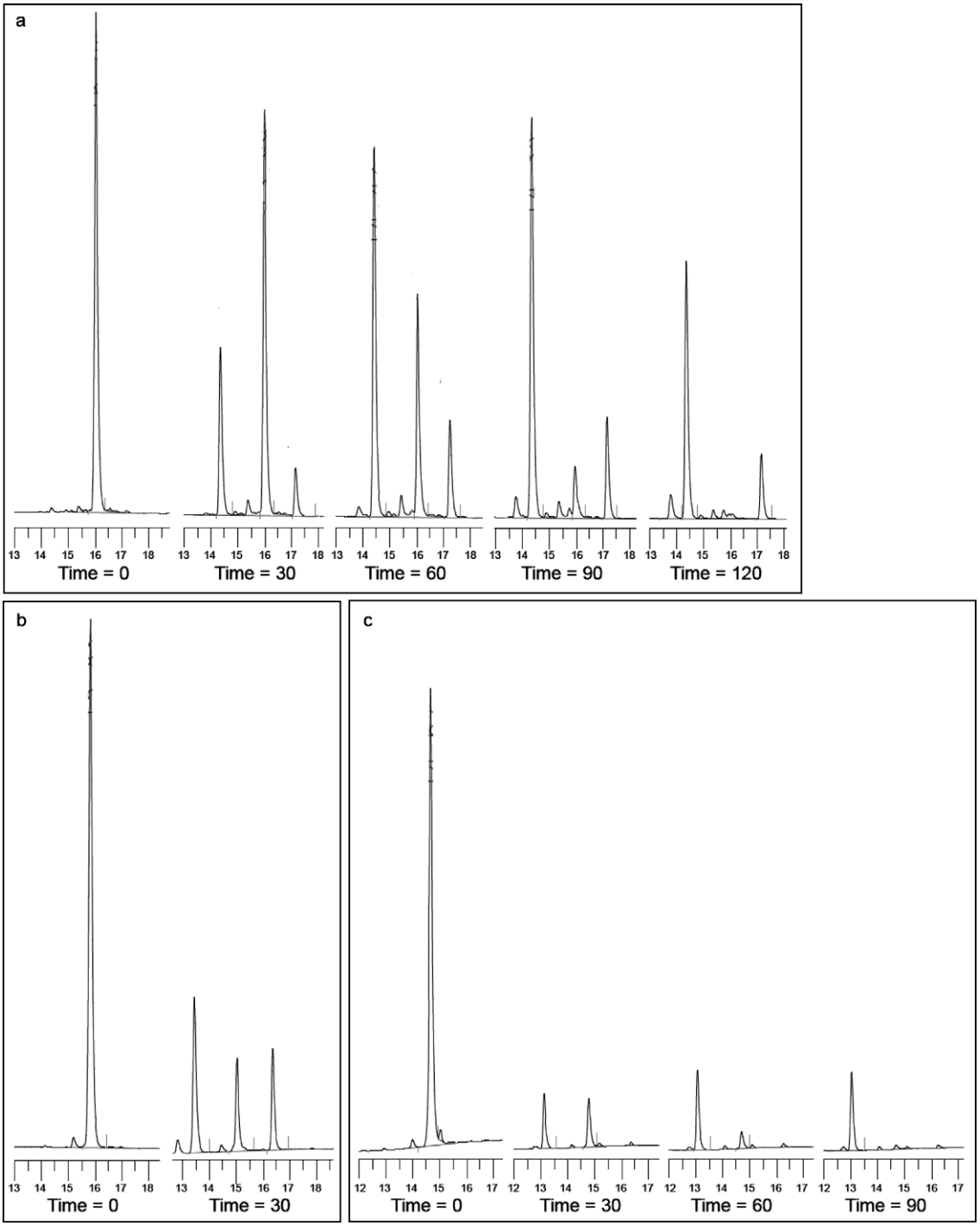


Figure 3. HPLC analysis of the degradation of the peptides by a mixture of trypsin and chymotrypsin. (a) PTR6154. (b) N-Me 1a. (c) N-Me 2a. Time = minutes from the addition of proteases.

with NMP (3 × 2 min). HATU coupling: Fmoc protected amino acids (1.5 equiv) were dissolved in NMP, DIPEA (1.5 equiv) and 1-hydroxy-7-aza-benzotriazole (HOAt) were added and the mixture was cooled to 0 °C. 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.5 equiv) was added and the mixture was pre-activated by mixing for 10 min, added to the resin, and shaken overnight at room temperature. The resin was washed with NMP (3 × 2 min). Microwave assisted HATU coupling: Fmoc protected amino acids (3 equiv) were dissolved in NMP. DIPEA (3 equiv) and HATU (3 equiv) were added, the mixture was pre-activated by mixing for 1 min and added to the resin. The reaction was conducted by microwave irradiation (25 W, 75 °C, 20 min) and the resin was washed with NMP (×3). BTC coupling: the resin was pre-washed with dibromoethane at 50 °C using water bath. Fmoc protected amino acids (5 equiv) were dissolved in dibromoethane. BTC (1.67 equiv) was added and the mixture was cooled to 0 °C. 2,4,6-collidine (14 equiv) was added dropwise and the mixture was pre-activated by stirring for 1 min, added to the resin, and shaken overnight at 50 °C. The resin was washed with CH_2Cl_2 (5 × 2 min). Reductive alkylation: the aldehyde (4 equiv) was dissolved in a solution of 1% acetic acid in NMP/MeOH 1:1 and then added to the resin for 15 min under

Table 9
Fragmentation of the peptides after degradation by trypsin and chymotrypsin

Name	Structure	Observed MH^+
PTR6154	H-Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	902.67
Fragment 1	H-Arg-Pro-Arg-Nva-Tyr-OH	690.43
Fragment 2	H-Dap-Hol-NH_2	231.20
Fragment 3	H-Arg-Pro-Arg-OH	428.66
Fragment 4	H-Nva-Tyr-Dap-Hol-NH_2	493.52
N-Me 1a	H-N(Me)Arg-Pro-Arg-Nva-Tyr-Dap-Hol-NH_2	916.45
Fragment 1	H-N(Me)Arg-Pro-Arg-Nva-Tyr-OH	704.55
Fragment 2	H-Dap-Hol-NH_2	231.25
Fragment 3	H-N(Me)Arg-Pro-Arg-OH	442.36
Fragment 4	H-Nva-Tyr-Dap-Hol-NH_2	493.63
N-Me 2a	H-Arg-N(Me)Ala-Arg-Nva-Tyr-Dap-Hol-NH_2	890.48
Fragment 1	H-Arg-N(Me)Ala-Arg-Nva-Tyr-OH	678.52
Fragment 2	H-Dap-Hol-NH_2	231.19
Fragment 3	H-Arg-N(Me)Ala-Arg-OH	416.32
Fragment 4	H-Nva-Tyr-Dap-Hol-NH_2	493.47

an argon stream. The resin was washed once with 1% acetic acid in NMP/MeOH 1:1 solution and $NaBH_3CN$ (4 equiv) was added under an argon stream. The reaction vessel was shaken for 3 h. The resin was washed with CH_2Cl_2 (5×2 min). Microwave assisted reductive alkylation: the resin was dissolved in 2 ml of 1% acetic acid and 5% dioxane in DMF. The aldehyde (4 equiv) was dissolved in 2 ml DMF and added to the solvated resin. The reaction was started by microwave irradiation (150 W, 80 °C, 2.5 min). $NaBH_3CN$ (4 equiv) was then added and then the reaction was continued with microwave irradiation (150 W, 80 °C, 6 min). The resin was washed twice with DMF. Coupling of bromo-acetic acid: Bromo-acetic acid (10 equiv) was dissolved in DMF. *N*,*N*′-Di-isopropylcarbodiimide (10 equiv) was added and the mixture was pre-activated by mixing for 20 min, and then added to the resin and shaken for 30 min. The procedure was repeated once more. The resin was washed with NMP (3×2 min). Coupling of amine: the resin was shaken with DMF at 50 °C using water bath. The amine (10 equiv) was dissolved in DMF. DIPEA (10 equiv) was added and the mixture was shaken for 1 min, added to the resin and shaken overnight at 50 °C. The resin was washed with NMP (3×2 min). *o*-NBS protection: *o*-NBS (4 equiv) was dissolved in NMP. 2,4,6-Collidine (10 equiv) was added and the mixture was shaken for 1 min. The mixture was added to the resin and shaken for 15 min. The procedure was repeated once more. The resin was washed with NMP (3×2 min). N^{α}-Methylation: DBU (3 equiv) was dissolved in NMP, the mixture was added to the resin and shaken for 3 min. Dimethylsulfate (10 equiv) was dissolved in NMP, added to the solvated resin and shaken for 2 min. The resin was washed once with NMP and the procedure was repeated once more. The resin was washed with NMP (5×2 min). *o*-NBS deprotection: Mercaptoethanol (10 equiv) and DBU (5 equiv) were dissolved in NMP. The mixture was added to the resin and shaken for 5 min. The procedure was repeated once more. The resin was washed with NMP (5×2 min). Cleavage: The resin was washed with CH_2Cl_2 (2×2 min) and dried under vacuum. A solution of 2.5% TDW and 2.5% triisopropylsilane in trifluoroacetic acid (TFA) was added and the reaction proceeded for 3 h at room temperature. The solution was separated by filtration and the resin was rinsed with neat TFA. The TFA mixture was treated with a cooled solution of ether/hexane 1:1 and the peptides were precipitated by centrifugation. The crude peptides were dissolved in acetonitrile/TDW 1:1 solution and lyophilized.

4.3. PKB/Akt assay

PKB/Akt kinase (HisΔPHPKBEEEFlag) was prepared as described by Klein et al., except that for routine screening the enzyme was only partially purified in one step on Ni-NTA agarose (Qiagen).[44] The radioactive kinase assay was done as described by Reuveni et al.,[2] except that the reaction mix comprised 50 mM Hepes pH 7.4, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate, 3-μM RPRTSSF peptide, 10 μM [γ32P-ATP (1 μCi/assay well), the inhibitory compound and 0.005 units HisΔPHPKBEEEFlag. For initial screening, the compounds were tested at 3–4 concentrations. Compounds that showed significant inhibition at 10 μM or less were retested and IC_{50} values determined using Graphpad Prism 5. PTR6154 was included in every assay as a standard.

4.4. Trypsin stability assay

The trypsin stability assay was conducted as described by Pakkala et al.[45] 400 μL of each peptide (1 mM) dissolved in 200 mM NH_4HCO_3 buffer solution (pH 8) were mixed with 1 μL of trypsin and chymotrypsin (porcine pancreas) solution (2.5 mg/ 1 ml). The peptides were incubated at 37 °C, 30 μL samples were taken every 30 min and mixed with 30 μL of 2% TFA and 30% ACN in water. Samples were analyzed by HPLC and MALDI-TOF MS.

Acknowledgments

A.L. was supported by grants from The European Commission (Prokinase Consortium), the Prostate Cancer Foundation (USA) and the Goldhirsh Foundation (USA). We would like to thank Professor Dr. Luis Moroder and Mrs. Elisabeth Weyher-Stingl from the Max-Planck-Institute of Biochemistry, Martiensried, Germany, for the Exact Mass analysis of all the compounds.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.031.

References and notes

1. Klein, S.; Levitzki, A. *Curr. Opin. Cell Biol.* **2009**, *21*, 185.
2. Reuveni, H.; Livnah, N.; Geiger, T.; Klein, S.; Ohne, O.; Cohen, I.; Benhar, M.; Gellerman, G.; Levitzki, A. *Biochemistry* **2002**, *41*, 10304.
3. Davies, S. P.; Reddy, H.; Caivano, M.; Cohen, P. *Biochem. J.* **2000**, *351*, 95.
4. Gumireddy, K.; Baker, S. J.; Cosenza, S. C.; John, P.; Kang, A. D.; Robell, K. A.; Reddy, M. V. R.; Reddy, E. P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1992.
5. Gumireddy, K.; Reddy, M. V. R.; Cosenza, S. C.; Nathan, R. B.; Baker, S. J.; Papathi, N.; Jiang, J. D.; Holland, J.; Reddy, E. P. *Cancer Cell* **2005**, *7*, 275.
6. Godl, K.; Wissing, J.; Kurtenbach, A.; Habenberger, P.; Blencke, S.; Gutbrod, H.; Salassidis, K.; Stein-Gerlach, M.; Missio, A.; Cotten, M.; Daub, H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15434.
7. Alfaro-Lopez, J.; Yuan, W.; Phan, B. C.; Kamath, J.; Lou, Q.; Lam, K. S.; Hruby, V. J. *J. Med. Chem.* **1998**, *41*, 2252.
8. Levitzki, A. *Acc. Chem. Res.* **2003**, *36*, 462.
9. Harris, T. E.; Persaud, S. J.; Saermark, T.; Jones, P. M. *Biochem. Soc. Trans.* **1995**, *23*, S187.
10. Litman, P.; Ohne, O.; Ben-Yaakov, S.; Shemesh-Darvish, L.; Yechezkel, T.; Salitra, Y.; Rubnov, S.; Cohen, I.; Senderowitz, H.; Kidron, D.; Livnah, O.; Levitzki, A.; Livnah, N. *Biochemistry* **2007**, *46*, 4716.
11. Naider, F.; Goodman, M.. In *Synthesis of Peptides and Peptidomimetics*; Goodman, M., Toniolo, C., Moroder, L., Felix, A., Eds.; Thieme: Stuttgart New York, 2002; Vol. E22a, p 1.
12. Ahn, J. M.; Boyle, N. A.; MacDonald, M. T.; Janda, K. D. *Mini-Rev. Med. Chem.* **2002**, *2*, 463.
13. Gante, J. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1699.
14. Simon, R. J.; Kania, R. S.; Zuckermann, R. N.; Huebner, V. D.; Jewell, D. A.; Banville, S.; Ng, S.; Wang, L.; Rosenberg, S.; Marlowe, C. K., et al *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 9367.
15. Stawikowski, M.; Stawikowska, R.; Jaskiewicz, A.; Zablotna, E.; Rolka, K. *Chembiochem* **2005**, *6*, 1057.
16. Miller, S. M.; Simon, R. J.; Ng, S.; Zuckermann, R. N.; Kerr, J. M.; Moos, W. H. *Bioorg. Med. Chem. Lett.* **1994**, 2657.
17. Wang, Y.; Lin, H.; Tullman, R.; Jewell, Jr. C. F.; Weetall, M. L.; Tse, F. L. S. *Biopharm. Drug Dispos.* **1999**, *20*, 69.
18. Cody, W. L.; He, J. X.; Reily, M. D.; Haleen, S. J.; Walker, D. M.; Reyner, E. L.; Stewart, B. H.; Doherty, A. M. *J. Med. Chem.* **1997**, *40*, 2228.

19. Mazur, R. H.; James, P. A.; Tyner, D. A.; Hallinan, E. A.; Sanner, J. H.; Schulze, R. *J. Med. Chem.* **1980**, *23*, 758.
20. Haviv, F.; Fitzpatrick, T. D.; Swenson, R. E.; Nichols, C. J.; Mort, N. A.; Bush, E. N.; Diaz, G.; Bammert, G.; Nguyen, A.; Rhutasel, N. S.; Nellans, H. N.; Hoffman, D. J.; Johnson, E. S.; Greer, J. *J. Med. Chem.* **1993**, *36*, 363.
21. Goodfellow, V. S.; Marathe, M. V.; Kuhlman, K. G.; Fitzpatrick, T. D.; Cuadrado, D.; Hanson, W.; Zuzack, J. S.; Ross, S. E.; Wieczorek, M.; Burkard, M.; Whalley, E. T. *J. Med. Chem.* **1996**, *39*, 1472.
22. Tonelli, A. *Biopolymers* **1976**, *15*, 1615.
23. Ron, D.; Gilon, C.; Hanani, M.; Vromen, A.; Selinger, Z.; Chorev, M. *J. Med. Chem.* **1992**, *35*, 2806.
24. Dechantsreiter, M. A.; Planker, E.; Matha, B.; Lohof, E.; Holzemann, G.; Jonczyk, A.; Goodman, S. L.; Kessler, H. *J. Med. Chem.* **1999**, *42*, 3033.
25. Wormser, U.; Laufer, R.; Hart, Y.; Chorev, M.; Gilon, C.; Selinger, Z. *EMBO J.* **1986**, *5*, 2805.
26. Laufer, R.; Wormser, U.; Friedman, Z. Y.; Gilon, C.; Chorev, M.; Selinger, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7444.
27. Bach, A. C.; Eyermann, C. J.; Gross, J. D.; Bower, M. J.; Harlow, R. L.; Weber, P. C.; Degrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 3207.
28. Teixido, M.; Albericio, F.; Giralt, E. *J. Pept. Res.* **2005**, *65*, 153.
29. Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. *Acc. Chem. Res.* **2008**, *41*, 1331.
30. Chan, W. C.; White, P. D. *Fmoc Solid Phase Peptide Synthesis*; Oxford University Press: Oxford, 2000.
31. Grand, V.; Aubry, A.; Dupont, V.; Vicherat, A.; Marraud, M. *J. Pept. Sci.* **1996**, *2*, 381.
32. Meyer, J. P.; Davis, P.; Lee, K. B.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **1995**, *38*, 3462.
33. St Hilaire, P. M.; Alves, L. C.; Herrera, F.; Renil, M.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Juliano, M. A.; Juliano, L.; Arevalo, J.; Meldal, M. *J. Med. Chem.* **2002**, *45*, 1971.
34. Nuss, J. M.; Desai, M. C.; Zuckermann, R. N.; Singh, R.; Renhowe, P. A.; Goff, D. A.; Chinn, J. P.; Wang, L.; Dorr, H.; Brown, E. G.; Subramanian, S. *Pure Appl. Chem.* **1997**, *69*, 447.
35. Park, M.-S.; Oh, H.-S.; Cho, H.; Lee, K.-H. *Tetrahedron Lett.* **2007**, *48*, 1053.
36. Falb, E.; Yechezkel, T.; Salitra, Y.; Gilon, C. *J. Pept. Res.* **1999**, *53*, 507.
37. Barda, Y.; Cohen, N.; Lev, V.; Ben-Aroya, N.; Koch, Y.; Mishani, E.; Fridkin, M.; Gilon, C. *Nucl. Med. Biol.* **2004**, *31*, 921.
38. Qvit, N.; Hatzubai, A.; Shalev, D. E.; Friedler, A.; Ben-Neriah, Y.; Gilon, C. *Biopolymers* **2009**, *91*, 157.
39. Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678.
40. Urban, J.; Vaisar, T.; Shen, R.; Lee, M. S. *Int. J. Pept. Prot. Res.* **1996**, *47*, 182.
41. Bondebjerg, J.; Xiang, Z.; Bauzo, R. M.; Haskell-Luevano, C.; Meldal, M. *J. Am. Chem. Soc.* **2002**, *124*, 11046.
42. Biron, E.; Chatterjee, J.; Kessler, H. *J. Pept. Sci.* **2006**, *12*, 213.
43. Lucjan, P.; George, N.; Harold, A. S. *Biopolymers* **1987**, *26*, 1587.
44. Klein, S.; Geiger, T.; Linchevski, I.; Lebendiker, M.; Itkin, A.; Assayag, K.; Levitzki, A. *Protein Expr. Purif.* **2005**, *41*, 162.
45. Miikka, P.; Can, H.; Pasi, S.; Jari, L.; Hannu, K.; Janne, W.; Ulf-Håkan, S.; Jouko, V.; Ale, N. *J. Pept. Sci.* **2007**, *13*, 348.