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Short Polybasic Peptide Sequences Are Potent Inhibitors of PC5/6 and PC7: Use of PS-SPCL as a Tool for the Optimization of Inhibitory Sequences

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Abstract

Positional Scanning-Synthetic Peptide Combinatorial Libraries (PS-SPCLs) are powerful molecular tools to identify enzyme substrate and potent inhibitory sequences while also providing crucial information about active site determinants. Previously, PS-SPCLs have been surveyed for furin, PC2, PC1/3 and PACE4 and proven efficient to identify potent peptidyl inhibitors in the low nanomolar range for furin and PC1/3. We report herein the screenings of non-amidated and acetylated hexapeptide PS-SPCLs for PC5/6A and PC7. The (L)-library surveys distinctively revealed that (L)-Arg, (L)-Lys and sometimes (L)-His in all six positions would generate the most potent inhibitors for both enzymes. Based on this clear polybasic preference, (L)-polyarginine peptides ranging from 4 to 9 residues were assayed. Inhibitory potency of these polybasic peptides increased with chain length, making nona-(L)-arginine a potent nanomolar inhibitor of PC5/6A and PC7 (K_i of 150 nM and 120 nM). PC5/6 and PC7 inhibition by nona-(L)-arginine was equivalent to that of furin (K_i of 114 nM) (Cameron et al., 2000). Nona-(D)-arginine was a more potent inhibitor of PC5/6 and PC7 than its levorotatory version (K_i of 19 nM and 81 nM), reminiscent of furin (K_i of 1.3 nM) (Kacprzak et al., 2004). Our data indicate that certain poly-arginine peptides represent potent inhibitors targeting PCs of the constitutive secretory pathway (furin, PC5/6 and PC7). We conclude that basic residues within PC peptide inhibitors might be responsible for targeting PCs in general and for inhibitory potency, but that select amino acid changes will be necessary to acquire true specificity toward a single PC.

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Introduction

PC5/6 and PC7 belong to the mammalian family of pro-protein convertases (PCs) that comprises seven calcium-dependant subtilisin/kexin-related serine endopeptidases. These are furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7. PCs are known to be at the center of a multitude of physiological events, being responsible for the activation of precursor proteins found in the secretory pathway that are essential to both the cell infrastructure and its functions. These precursor proteins include zymogens, pro-receptors, pro-growth factors, and pro-hormones (Fugere and Day, 2005; Rockwell et al., 2002). Since PCs are such valuable assets for the cell, their processing functions are also often taken advantage of by opportunistic infections or pathophysiological conditions leading to severe deleterious effects. Examples include the maturation of viral glycoproteins, e.g. HIV-1 gp160, SARS-cov spike, and hepatitis B e antigen (Bergeron et al., 2005; Chekanov et al., 2006; Johannsen et al., 2004; Kibler et al., 2004; Messageot et al., 2003; Richards et al., 2006), and of oncogenic proteins like matrix metalloproteases (Bassi et al., 2000; Mercapide et al., 2002; Remacle et al., 2006), growth factors (Dubois et al., 2001; Khatib et al., 2001; Siegfried et al., 2003; Stawowy et al., 2004), and adhesion molecules (Muller et al., 2004; Posthaus et al., 1998; Posthaus et al., 2003). In fact, some PCs have been qualified as pro-oncogene "master switches" given their core implication in cancer initiation and progression, i.e. cellular transformation, acquisition of the tumorigenic phenotype, invasion and metastasis (Bassi et al., 2005). In light of their clear pathophysiological and clinical relevance, PCs have emerged in the last years as potential pharmacological targets and thus, current research is becoming increasingly focused on the design of PC inhibitors and the association of each PC with endogenous substrates responsible for pathological phenotypes.

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Based on both *in vivo* and *in vitro* studies, it is evident that all PCs process substrates C-terminal to pairs of basic residues, such as Arg-Arg↓ and Lys-Arg↓, and less often at single basic residues. The furin recognition motif for cleavage of substrates is well defined and is now known to extend N-terminally to include additional basic residues that make catalysis more efficient, i.e. ^{P6}R-x-R-x-K/R-R^{P1}↓ (for nomenclature see (Fugere and Day, 2005)). The recognition motif of the other PCs, including PC5/6 and PC7, has been far less investigated and still remains poorly understood. Although many studies demonstrated a significant level of functional redundancy among some PCs and for some substrates (Roebroek et al., 2004), distinct cleavage patterns are also observed. For example, PC2 can process substrates with a P1 Lys and most other PCs are apparently less stringent for basic residues afar the C-terminal pair (Duckert et al., 2004; Rouille et al., 1995; Thomas et al., 1991). Since PCs recognition sequences are reflective of their active site structure, distinct molecular determinants could be exploited for the design of uniquely specific PC peptide inhibitors.

In the hope to identify these distinctive molecular determinants in the active sites of PC5/6 and PC7, we have screened Positional Scanning Synthetic Peptide Libraries (PS-SPCLs) of hexapeptides. These libraries have previously been screened for furin, PC1/3, PC2 and PACE4 and have led to the development of a potent inhibitor for furin, poly-arginine (Cameron et al., 2000), and to the identification of a sequence potent for the inhibition of PC1/3, LLRVKR (Apletalina et al., 1998). Subsequently, the latter was found embedded in the endogenous endocrine protein precursor ProSAAS (Fricker et al., 2000). Our results show that basic residues in all positions are most favored by both PC5/6 and PC7, and that polybasic sequences are highly potent inhibitors of these two enzymes in the same range of concentration as for furin. Thus, poly-arginine peptides are general inhibitors of the PCs of the constitutive secretory pathway and not furin-specific inhibitors. We conclude that interpretation of results in studies using poly-

arginine peptides for *ex vivo* or *in vivo* furin activity blockade should take under account that additional PCs are in fact affected.

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Material and Methods

Materials **-** Pyro-Glu-Arg-Thr-Lys-Arg-AMC (pERTKR-AMC) was purchased from Peptides International, Inc (Louisville, KY). Positional Scanning-Synthetic Peptide Combinatorial Libraries (PS-SPCLs) were synthesized at the Torrey Pines Institute for Molecular Studies (San Diego, CA) using simultaneous multiple peptide synthesis methodology as previously described (Houghten et al., 1991; Pinilla et al., 1995; Pinilla et al., 1994) and supplied at concentrations of 5 or 10 mg/mL. Two PS-SPCLs were screened for the inhibition of PC5/6 and PC7. (L)- and (D)-hexapeptide PS-SPCLs both consisted of 120 hexapeptide mixtures, N-terminally acetylated and C-terminally amidated, divided into 6 groups corresponding to each position within a hexapeptide sequence. For each position, 20 mixtures were surveyed, each of which was defined by 1 of the 20 natural L- or D-amino acids. All remaining positions were undefined and occupied by any amino acid except cysteine.

Production of Recombinant Human PC5/6 and PC7 **-** Recombinant PC5/6A and PC7 preparations were produced by secreted expression by Schneider 2 cells in the conditioned medium and purified using ultrafiltration and fast performance liquid chromatography methods, as previously described (Fugere et al., 2002). Briefly, 2L of conditioned media was concentrated to 100 mL using tangential flow ultrafiltration (Pellicon II, Millipore, MA) against a 30-kDa cut-off Biomax membrane and the buffer was exchanged to 20 mM Bis-Tris pH 6.5, 1 mM $CaCl_2$. The concentrate was loaded on an anion exchange column (MonoQ HR 10/10, Pharmacia Biotech, NJ) and eluted using a linear gradient of 0-60% 1M NaCl. Active fractions were pooled, made 1 M $(NH_4)_2SO_4$, loaded on an hydrophobic column (HIC 6FF high sub, Pharmacia Biotech, NJ) and eluted with a 100-0% 1M $(NH_4)_2SO_4$ gradient. Last, active PC5/6 or PC7 was isolated by

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gel filtration (Superdex 200 26/60, Pharmacia Biotech, NJ), concentrated and frozen at -80°C in aliquots until used.

Hexapeptide Libraries Screens **-** For both (L)- and (D)-hexapeptide PS-SPCLs, thoroughly mixed hexapeptide mixtures were individually pre-incubated with PC5/6 or PC7 (1.5-3 units/well) at a final concentration of 1 and 0.5mg/mL. Reactions were made 20mM Bis-Tris pH6.5, 1mM $CaCl_2$ with the addition of 0.1% Triton when mentioned, and took place in 96-wells white microplates in a volume of 90µL for 30 minutes at 37°C. Substrate (pERTKR-AMC) was then added to a final concentration of 100µM for PC5/6A and 250µM for PC7. The total volume of each reaction was 100µL. Real-time kinetic progression of the release of fluorescent AMC was monitored for 30 to 60 minutes at 37°C using Ascent 2.4 software and Fluoroskan fluorometer (Labsystems, Inc) with EX/EM wavelengths of 380/460nM. The rate of hydrolysis of the substrate was determined relative to control samples lacking inhibitory peptides for which kinetic progression proceeded linearly during this period of time. Both libraries were surveyed independently at least twice for each enzyme and sample reactions were all done in triplicates.

Calculations and Data Interpretation- Crude data obtained from screening the libraries in this study are shown as percentage of maximum enzymatic activity in sets of histograms. Preferred amino acids for each position are then listed in tables. The contribution to inhibition of these preferred amino acids is shown in these tables as a percentage of inhibition that excludes the average enzyme sensitivity. The average enzyme sensitivity at a specified position is a normalization of the library that corresponds to the average percentage of inhibition generated by all 19 mixtures (excluding mixtures with an activating effect), and could be reflective of the enzyme specificity spectrum at that position or of the heterogeneous nature of the mixtures. The

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contribution to inhibition of an amino acid at a specified position is equal to the maximum activity minus the average enzyme sensitivity at that position and the remaining enzyme activity.

Determination of K_i values for PC5/6A and PC7 Inhibition by Synthetic peptides - Information provided by the screening of the hexapeptide PS-SPCLs led to the evaluation of the inhibitory potency of specific peptide sequences of various lengths against both enzymes. Synthetic peptide synthesis was performed by the Louisiana State University Health Sciences Center Core Laboratories and identity was verified by mass spectrum analysis. All peptides followed competitive kinetic behavior. To evaluate the apparent inhibition ($K_{i\ (app)}$), both enzymes were incubated with decreasing concentrations of the different inhibitory peptides, ranging from 800µM to 0.1nM, in activity buffer (20mM Bis-Tris pH 6.5, 1mM $CaCl_2$) and in the presence of 100µM or 250µM substrate (pERTKR-AMC), for PC5/6 and PC7 respectively. Residual activity was measured online for 1 hour at 37°C with continuous shaking by reading resulting fluorescence with a Gemini XS microplate fluorometer (Molecular Devices, CA) EX/EM/CO 370/460/435nM. Quantative data analysis was done using four-parameter logistic (SoftMaxPro4, Molecular Devices, CA) and real inhibition constants (K_i) were determined using the method and K_m values previously described (Fugere et al., 2002).

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Results

Screening of (L)-hexapeptide PS-SPCL **-** For both PC5/6 and PC7, screens at a final assay peptide concentration of 1 mg/ml were consistent, showed enough discrimination between overall mixtures effect on enzyme activity and inhibitory preferences that was sought for, and led to the clear identification of PC5/6 and PC7 preferences at all six positions.

In Figure 1, we show the results compiled from these screens as a percentage of maximum enzyme activity. The lower the histogram bar, the greater the inhibition. An overall comparison of the effect of all (L)-hexapeptide PS-SPCL mixtures shows that PC5/6 was much more sensitive in general to these mixtures than was PC7 (Figure 1A and B). This effect has been normalized as described in Material and Methods. We identified the amino acids generating the greatest reduction of activity and listed them in Table 1. PC5/6 showed clear preferences for basic residues at all positions (Table 1A). At P_1 (L)-Arg (37%) was favored over (L)-His (21%) and (L)-Lys (8%). At P_2, (L)-Arg (25%) and (L)-Lys (23%) were preferred to (L)-His (15%). At P_3, the pattern preference for basic residues was similar to P_2, but the inhibition was less prominent with (L)-Lys (18%), (L)-Arg (17%) and (L)-His (11%). At P_4, (L)-Arg (30%) was favored over (L)-His (20%) and (L)-Lys (15%), similar to P_1. At P_5, (L)-Arg (18%) was preferred to (L)-Lys (13%) and (L)-His (13%). Finally, at P_6, (L)-Arg (19%) and (L)-Lys (16%) were preferred to (L)-His (8%). In positions P_5 and P_6, although basic residues were most preferred, (L)-Trp stood out somewhat as well. This could suggest that PC5/6 active site can offer more flexibility at these positions by allowing an alternative binding conformation for a hydrophobic residue to fit, but could also suggest different binding configurations of the peptides in these mixtures.

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As seen in Figure 1B, PC7 was overall less sensitive to the (L)-hexapeptide PS-SPCL mixtures and exhibited far more discrimination than PC5/6 at the same peptide concentration. PC7 preferences were much more drastic and showed less flexibility for all six positions. PC7 favored basic residues at all positions and, in contrast with PC5/6, no hydrophobic residues stood out at the far positions P_5 and P_6 (Figure 1B). In Table 1B, we list PC7 preferred amino acids and their contribution to inhibition. At positions P_1 and P_2, the (L)-Arg and (L)-Lys mixtures generated strong inhibition (69% and 63% for P_1 and P_2 respectively for both amino acids). At P_3, (L)-Arg (60%) and to a lesser extent (L)-His (32%) were markedly preferred, but not (L)-Lys. At positions P_4, P_5 and P_6, (L)-Arg and (L)-Lys, were clearly favored, generating between 66% and 95% of the observed inhibition (Table 1B).

In contrast to results obtained with PC5/6, a few peptide mixtures showed slight to considerable PC7 activity enhancing effects (Figure 1B). Many of these residues were negatively charged amino acids. The most remarkable ones are (L)-Asp and (L)-Phe at P_6, (L)-Asp at P_5, (L)-Asp, (L)-Glu and (L)-Gly at P_4, and (L)-Asp and (L)-Glu at P_3. It is possible that these mixtures containing random peptide sequences with a fixed negative residue at the specified position can bind to a putative allosteric site separate from the active site of PC7, thus causing an enhancement in activity. However, this idea requires further work in support.

Screening of the (D)-Hexapeptide PS-SPCL - The same approach was taken for the screening of the (D)-hexapeptide PS-SPCL. Figure 2 shows the compiled data for these screens in sets of histograms, while Table 2 lists the contribution to inhibition of the most effective (D)-amino acids. The general effect on activity by these mixtures was less important than for the (L)-hexapeptide PS-SPCL, but discrimination was also much more difficult to attain for both PC5/6 and PC7. Figure 2A shows that PC5/6 favors (D)-Arg at all positions and that the second and

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third best (D)-amino acids are (D)-Lys and (D)-His, except for P_1 where (D)-Lys had no effect. Comparison of Table 2A to Table 1A shows that the contribution to inhibition of PC5/6 by (D)-Lys and (D)-His at all effective positions (~10%) is roughly half of their levorotatory versions, but that (D)-Arg remains as efficient as (L)-Arg in all positions (36% for P_1 and ~ 25% for P_2-P_6). In contrast to the (L)-hexapeptide PS-SPCL, no hydrophobic (D)-amino acids were preferred at the far P_5 and P_6 positions (Figure 2A).

For PC7, Figure 2B shows that inhibitory potency of the mixtures was much more variable and that hydrophobic (D)-amino acid peptide mixtures were often more efficient for inhibition. At P_1, hydrophobic (D)-Phe (19%), polar (D)-Tyr (16%), and positively charged (D)-Arg (11%) were favored. At P_2, (D)-Arg (39%) and (D)-Tyr (37%) were slightly favored over several effective hydrophobic residues, including (D)-Phe (26%), (D)-Val (20%) and (D)-Ile (18%), but not (D)-Leu. At P_3, several hydrophobic residues were preferred, including (D)-Phe (31%), (D)-Leu (30%), (D)-Val (27%) and (D)-Ile (26%), and were more efficient than (D)-Arg (18%) and (D)-Tyr (18%). At position P_4, non-polar (D)-Val and polar (D)-Tyr (24%) were preferred, followed by hydrophobic (D)-Ile (20%), (D)-Leu (16%) and (D)-Phe (13%), and (D)-Arg had not effect. At P_5, preferences were strictly for the hydrophobic residues but (D)-Val. Finally, at P_6, (D)-Phe (20%), (D)-Ile (17%) and (D)-Tyr (17%) were preferred, while (D)-Arg (13%) was as efficient as (D)-Leu (12%).

Interestingly, the addition of a small amount of detergent (0.1% Triton) to the screening assay reduced the observed variability of the (D)-hexapeptide PS-SPCL for PC7 (Figure 3). It also reduced inhibitory discrimination but, it re-established PC7 preferences for basic (D)-amino acids at all positions, just like for the (L)-hexapeptide PS-SPCL. (D)-Arg (37%), (D)-Lys (36%) and (D)-His (21%) were preferred at position P_1. (D)-Arg (~12%) , but not (D)-Lys and (D)-His, was preferred at all other positions (Figure 3 and Table 2C).

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Inhibition of PC5/6 and PC7 by Poly-(L)-arginine and Poly-(D)-arginine peptides - PS-SPCL screens are only indicative of a general pattern of specificity and thus the evaluation of single peptide inhibitors are required to assess efficiency and confirm specificity of inhibition. The best or best two amino acids at each position as compiled in Table 1 and Table 2 were considered to compose hypothetical inhibiting (L)- and (D)- hexapeptide sequences for PC5/6 and PC7 (Table 3). For both PC5/6 and PC7, a poly-arginine hexapeptide or a Arg/Lys hexapeptide were the most logical choice. Given the clear preferences of PC5/6 and PC7 for basic residues at all six positions, we tested a series of synthetic polybasic peptides ranging from four to nine (L)-arginine for potency of inhibition. Table 4 shows that all poly-(L)-arginine peptides were potent inhibitors of PC5/6 and PC7. Figure 4 shows that inhibition potency increased with peptide length. Tetra-(L)-arginine was the least potent inhibitor with K_i values in the low micromolar range, while nona-(L)-arginine was the most efficient inhibitor with K_i values in the lower-mid nanomolar concentrations for both enzymes.

For PC5/6, the addition of a fifth Arg residue to tetra-(L)-arginine (K_i of 2.5 μM) to make penta-(L)-arginine (K_i of 450 nm) represented the greatest increase in potency of the polybasic peptide series, i.e. 5 times K_i value decrease. Intermediate lengths of six, seven and eight (L)-Arg residue peptides were equivalent in potency to that of penta-(L)-arginine. The addition of a ninth Arg residue to octa-(L)-arginine (K_i of 450 nM) increased potency three more times (K_i of 150 nM). For PC7, the greatest increase in potency was also generated by the addition of a fifth Arg residue to tetra-(L)-arginine (K_i value of 6 μm) to make penta-(L)-arginine (Ki of 1.1μM), i.e. 6 times K_i value decrease. The addition of a sixth Arg residue had no effect (K_i of 1.05 nM). The addition of a seventh increased potency by three times (K_i of 312 nM). Addition of the eighth and ninth Arg residues generated both an increase in potency of 0.5 times (K_i values of 200 and 120 nM, respectively). Although hexa-(L)-arginine was twice as potent on PC5/6 (K_i of 430 nM) than

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PC7 (K_i of 1050 nM), nona-(L)-arginine inhibition potency for PC5/6 was very similar to PC7 (K_i values of 150 and 120 nm, respectively).

Inhibition of PC5/6 and PC7 by hexa-(D)-arginine and nona-(D)-arginine peptides was also evaluated. The K_i values presented in Table 4 show that for PC5/6, inhibition by hexa-(D)-arginine was more efficient than by hexa-(L)-arginine (K_i values of 265 and 430 nm, respectively). In contrast, inhibition of PC7 by hexa-(D)-arginine was less efficient than by hexa-(L)-arginine (K_i values of 1875 and 1050 nm, respectively). For both PC5/6 and PC7, inhibition by nona-(D)-arginine was much more efficient than any other peptide evaluated in this study. For PC5/6, nona-(D)-arginine was 8 times more efficient than nona-(L)-arginine and 13 times more efficient than hexa-(D)-arginine. For PC7, nona-(D)-arginine was 1.5 times more efficient than nona-(L)-arginine and 23 times more efficient than hexa-(D)-arginine. These results suggest that both peptide length and amino acid orientation affect potency of inhibition.

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Discussion

Basic (L)-amino acids at all positions are preferred by both PC5/6 and PC7 - PC5/6 and PC7 exhibited an almost identical pattern of preference to that of furin when screening the (L)-hexapeptide combinatorial library (Cameron et al., 2000). Overall, both enzymes' active sites demonstrated a net preference for binding basic residues stretching from the S_1 to S_6 subsite pockets, as observed for furin (Henrich et al., 2003). This conclusion correlates well with the distribution of negative charges in furin active site as determined by the crystal structure (Henrich et al., 2003) and in PC5/6 active site as observed by modeling studies (Henrich et al., 2005). However, these data correlate only partially with the modeling studies of the PC7 active site (Fugere and Day, 2005; Henrich et al., 2005), where the S_6 subsite pocket lacks one of the negative amino acids found in furin (Glu^{230}) and PC5/6 (Asp^{249}). This difference should offer an option for more flexibility for other amino acids to fit at the S_6 subsite pocket of PC7. In the screens for PC5/6, but not for PC7, (L)-Trp came out third at P_1 and fourth at the P_4, P_5 and P_6 positions in terms of inhibitory efficiency. Given the low probability that hydrophobic residues actually bind efficiently in the P_1 and P_4 subsite pockets, we suggest that these mixtures with a fixed (L)-Trp at these positions possibly bind to one or more sites away for the active site, at so called "exosites" (Apletalina et al., 2000; Bode and Huber, 1992).

In a previous comparative study, we evaluated the potency and specificity of inhibition of 12 amino acid C-terminally-derived prodomain peptides (propeptides). We showed that these propeptides are sufficient for inhibition of PCs although they exhibit a lack in specificity (Fugere et al., 2002). The most obvious example of this is the cross-inhibition capability of furin and PC5/6 by their respective propeptides. These propeptides differ by only three residues, but these distinct amino acids conserve their charge or uncharged nature (furin propeptide

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LEQQV**A**K**R**RTKR^{P1}; PC5/6 propeptide **IQ**QQV**V**K**K**RTKR^{P1}). The propeptide of furin could inhibit furin with a K_i value of 184 nm and PC5/6 with a K_i value of 27 nm. Similarly, the propeptide of PC5/6 could inhibit furin with a K_i value of 166 nm and PC5/6 with a K_i value of 23 nm. In contrast, the PC7 propeptide (**HSE**Q**RLL**^{P6}**R**R**A**KR^{P1}), which contains several distinctive amino acids including a Lys^{P6} → Leu^{P6} substitution, was a poor inhibitor of furin (K_i of 1μM) but was still potent on PC5/6. Thus it would seem that even though PC5/6 can accommodate a hydrophobic residue in S_6, furin and PC5/6 are still very much related both in terms of evolution of their prodomain and of inhibitory binding preferences at their active site. Even if PC7 propeptide is quite distinct from that of furin and PC5/6 and is efficient for the inhibition of PC7, basic residues are still markedly preferred (or more efficient for inhibition) by all three PCs. Phylogenic tree alignment analysis of the PCs shows that mouse Furin, PC4, PC5/6 and PACE4 have in fact a closer evolutionary relationship than PC7, which seems to have diverged from a common ancestor of the PCs earlier than the other PCs (Seidah et al., 1998).

The uniform preference for basic residues at all positions for furin, PC5/6 and PC7 contrasts with the preferences revealed from the screenings for PC1/3 and PC2 (Apletalina et al., 1998). PC1/3 presented a strong preference for (L)-Arg in positions P_1 and P_4, (L)-Lys in P_2 and (L)-Leu in P_6. PC2 showed a preference for (L)-Arg only in positions P_1 and P_4, and no clear consensus for the other positions. This extensive biochemical comparison of the binding preferences at the reactive site of these PCs underlines the close similarities found within the active sites of the PCs of the constitutive secretory pathway (furin, PC5/6 and PC7) as well as their distinction from the PCs of the regulated secretory pathway (PC1/3 and PC2). Among other conclusions, it will be helpful to consider novel approaches to identify unique molecular determinants as they will be necessary to achieve the sought specificity of inhibition of each PC.

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Basic (D)-amino acids combinatorial peptide mixtures are efficient for the inhibition of PC5/6 and PC7 **-** PC5/6 preferences for (D)-Arg in all positions were nearly identical to that of the (L)-hexapeptide PS-SPCL screen. Also, the contribution to inhibition of basic (D)-amino acids was comparable to basic (L)-amino acids, suggesting a similar mechanism of inhibition or a disregard for global amino acid orientation. The screening of this library with detergent for PC7 showed that basic residues such as (D)-Arg in all positions were also preferred by PC7. Again, these data follow the same pattern than observed for furin (Cameron et al., 2000) and suggest that the proper use of (D)-residues can be envisioned for the design of more stable peptide inhibitors for most of the PCs.

Poly-(L)- and (D)-arginine peptides are potent inhibitory peptides of PC5/6 and PC7 **-** Based on the data retrieved from the screens, polybasic sequences were clearly the only logical choice for the optimal inhibition of both PC5/6 and PC7 (Table 3). Indeed, similar to furin, both poly-(L)- and poly-(D)-arginine peptides are potent inhibitors of PC5/6 and PC7, have a competitive behavior (data not shown) and have an inhibition potency that increases with polybasic peptide length (Table 4). It has previously been shown that PC2 is not inhibited by any polyarginine peptides and that PC1/3 is only affected at relatively high polyarginine peptide concentrations (K_i values ranging from 4 to 14 μM) (Cameron et al., 2000). PACE4 inhibition by polyarginine peptides was also addressed and resembles closely the response pattern observed for PC5/6 and PC7. In fact, when comparing furin, PACE4, PC5/6 and PC7, an increase in length of (L)-polybasic peptides does both increase potency and tends to reduce specificity (Figure 4A).

Some distinctions in the sensitivity to inhibition can be observed among these PCs for some of the polyarginine peptides, but in general the range of inhibition potency is the same. Overall, polyarginine peptides of (L)- or (D)-amino acids are more efficient for the inhibition of furin. For

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example, hexa-(L)-arginine was more potent for furin and less for PC5/6, PACE4 and PC7 (in that order). Hexa-(D)-arginine inhibition potency remains in the same range as for hexa-(L)-arginine for all these four enzymes. Nona-(L)-arginine was the best (L)-polybasic peptide, but its inhibition potency is equivalent for all four PCs. Nona-(D)-arginine is a much better inhibitor than its levorotatory version for furin, PC5/6 and PC7, but its potency is low-nanomolar for all three enzymes (PACE4 was not evaluated). Thus, it would seem that neither polybasic peptide length increase or (L)- to (D)-amino acid substitutions really generate significant specificity of inhibition among these PCs.

PS-SPCLs screens leads to strategies for the design specific PC inhibitors **-** This comparative study provides us with templates and strategies on which to work in order to achieve high potency and high individual specificity in the design of PC peptide inhibitors. The fact that, for PC5/6, a greater increase in potency was observed when adding a fifth (P_5) and a ninth (P_9) Arg residue could suggest that these positions account for much of the potency of the polybasic sequences, while the P_1, P_2 and P_4 would mainly be responsible to ensure that the peptide targets a PC. Additionally, these data could suggest that distinct inhibitory peptides could be designed by shuffling the positions P_6, P_7 and P_8 in order to increase specificity to PC5/6, as these positions did not contribute significantly to the potency of inhibition. Furin had a greater increase in potency when adding a fifth (P_5) and a sixth (P_6) Arg residue and no increase for an eighth Arg residue, while PACE4 had a greater increase by adding a fifth (P_5) Arg residue and a gradual doubling increase in potency for each additional basic residues (Cameron et al., 2000). In contrast, for PC7, we observed a greater increase in potency when adding a fifth (P_5), seventh (P_7) and ninth (P_9) Arg residue, and no increase for the sixth and eighth Arg residues. Thus, shuffling of the P_6 and P_8 positions might lead to inhibitory peptides with higher specificity

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toward PC7. In fact, the PC7 propeptide most drastic distinctions are a P_6 Leu instead of Arg, and a P_8 Arg instead of Val when compared to the furin and PC5/6 propeptides (Fugere et al., 2002).

The summation of all the studies where PS-SPCLs have been screened for the inhibition of PCs allows one to appreciate and compare the binding tendencies of PCs and possibly to better understand the role played by each PC in the binding of natural substrates or inhibitors. Polyarginine peptides have already been used in several *ex vivo* and *in vivo* studies to block pathophysiological events. For example, hexa-(L)-arginine has been used to inhibit the processing of gp160 in the TGN and to suppress productive human immunodeficiency virus type 1 (HIV-1) infection of T-cells and macrophages *ex vivo* without indication of toxicity (Kibler et al., 2004). In addition, hexa-(D)-arginine was shown to be non-cytotoxic and to effectively block the maturation of *Pseudomonas Aeruginosa* exotoxin A (PEA) in CHO cells, as well as to significantly increase the survival rate of mice treated with PEA when administered *i.v*. (Sarac et al., 2002). Although these studies are great advances for the use of PC blocking agents in therapeutic applications, in most cases, the data obtained was interpreted as if only furin's activity was blocked. We believe that certain polyarginine peptides, including nona-(D)-arginine, are interesting compounds for the blockade of PC activity in therapeutic strategies, but only if the treatment of the pathological condition requires the inhibition of several PCs of the constitutive sercretory pathway, i.e. furin, PACE4, PC5/6 and PC7. For other pathophysiological situations where only one PC is implicated, uniquely specific inhibitory compounds will have to be designed. Choice of strategy will also depend on the ability to deliver a PC inhibitor specifically to the organ and cells whose pathological phenotype calls for the blocking of a determined PC process. Thus, the use of a combination of uniquely specific inhibitors for each PC could have the advantage of being used in situations where cell-specific targeting is not feasible.

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Figure Legends

Figure 1. Screening of (L)-hexapeptides PS-SPCL for *in vitro* inhibition of PC5/6 (A) and PC7 (B) activity. Each mixture is defined by a specified (L)-amino acid for one of the six positions of the hexapeptide sequence. The remaining five positions are composed of a mixture of 19 (L)-amino acids (all except cysteine). For example, at the panel "Amino acid in P1", only the P_1 position is defined and the other positions are random: Ac-X-X-X-X-X-P_1-NH_2. Inhibition or activation of enzymatic activity in the presence of peptide mixtures is given as a percentage of maximum rate relative to that of controls. The most inhibitory amino acids for each position are shaded in black.

Figure 2. Screening of (D)-hexapeptides PS-SPCL for *in vitro* inhibition of PC5/6 (A) and PC7 (B) activity. Refer to Figure 1.

Figure 3. Screening of (D)-hexapeptides PS-SPCL in presence of detergent for *in vitro* inhibition PC7 activity. Refer to figure 1. Screening assay conditions include 0.1% Triton.

Figure 4. Inhibition of Furin, PACE4 PC5/6, PC7 by variable length of poly-(L)-arginine peptides (A) and poly-(D)-arginine peptides (B). Graphics showing the effect of adding arginine residues to polyarginine peptides on the inhibition of PCs of the constitutive secretory pathway. (A) Addition of (L)-arginine residues to tetra-(L)-arginine causes an increase of the inhibition potency, but tends to reduce inhibitory specificity. (B) Nona-(D)-arginine is more potent then hexa-(D)-arginine. Increased potency of nona-(D)-arginine is greater for furin.

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Table 1. Preferred (L)-amino acids at each position by PC5/6 (A) and PC7 (B). Listing of the preferred amino acids by each enzymes and the contribution to inhibition (%) that is attributable to the amino acid at the specified position. Data has been normalized as described in Material and Methods. Only the most significant residues are presented (see Figure 1A and B). Based on these data, optimal sequences of peptides to evaluate for inhibition were established.

A

(L)-hexapeptide PS-SPCL for PC5/6	P_6	P_5	P_4	P_3	P_2	P_1
H	8	13	20	11	15	21
K	16	13	15	18	23	8
R	19	18	30	17	25	37
W	12	7	-	-	-	-

B

(L)-hexapeptide PS-SPCL for PC7	P_6	P_5	P_4	P_3	P_2	P_1
H	-	-	-	32	-	-
K	95	66	97	-	63	69
R	91	78	75	60	63	69

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Table 2. Preferred (D)-amino acids at each position by PC5/6 (A) and PC7 (B and C). Refer to table 1. Only the most significant residues are presented (see Figure 2A and B, and Figure 3).

A

(D)-hexapeptide PS-SPCL for PC5/6	P_6	P_5	P_4	P_3	P_2	P_1
H	9	6	8	7	9	10
K	8	9	7	10	10	-
R	22	19	28	24	20	36

B

(D)-hexapeptide PS-SPCL for PC7	P_6	P_5	P_4	P_3	P_2	P_1
F	20	35	13	31	26	19
I	17	22	20	26	18	-
L	12	22	16	30	-	-
R	13	-	-	18	39	11
V	-	-	26	27	20	-
Y	17	19	24	18	37	16

C

(D)-hexapeptide PS-SPCL for PC7 (with detergent)	P_6	P_5	P_4	P_3	P_2	P_1
K	-	-	-	-	-	36
R	10	12	10	18	10	37
H	-	-	-	-	-	21

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Table 3. Best inhibitory peptide sequences. A list of the best hexapeptide sequences was established for PC5/6 and PC7, as deduced from the screenings of (L)-and (D)-hexapeptide PS-SPCLs. The average inhibitory potency is a non-synergetic prediction of contribution to inhibition potency at 1mg/ml concentration.

Hexapeptide sequences for PC5/6	**Average potency**
P_6 P_5 P_4 P_3 P_2 P_1	
(L)-Arg-Arg-Arg-Lys-Arg-Arg	25%
(L)-Arg-Arg-Arg-Arg-Arg-Arg	24%
(D)-Arg-Arg-Arg-Arg-Arg-Arg	47%
Hexapeptide sequences for PC7	
(L)-Lys-Arg-Lys-Arg-Lys/Arg-Lys/Arg	77%
(L)-Arg-Arg-Arg-Arg-Arg-Arg	73%
(L)-Lys-Lys-Lys-Lys-Lys-Lys	25%
(D)-Phe-Phe-Val-Phe-Arg-Phe	28%
(D)-Phe-Phe-Phe-Phe-Phe-Phe	28%

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Table 4. Poly-(L)-arginine (A) and Poly-(D)-arginine (B) peptide inhibitory potency on PC5/6 and PC7. K_i values (nM) were obtained by computing the rate of hydrolysis of pERTKR-AMC by PC5/6 and PC7 in the presence of various concentrations of inhibitory peptides (as described in Materials and Methods). Data for furin and PACE4 are also provided for comparison (Cameron et al., 2000; Kacprzak et al., 2004). Differences in assay ionic strength are noted: Furin and PACE4 assays for poly-(L)-arginine peptides and hexa-(D)-arginine were done at 5 mM $CaCl_2$ (Cameron et al., 2000); Furin and PACE4 assays for nona-(D)-arginine were done at 0.1 mM $CaCl_2$ (Cameron et al., 2000; Kacprzak et al., 2004).

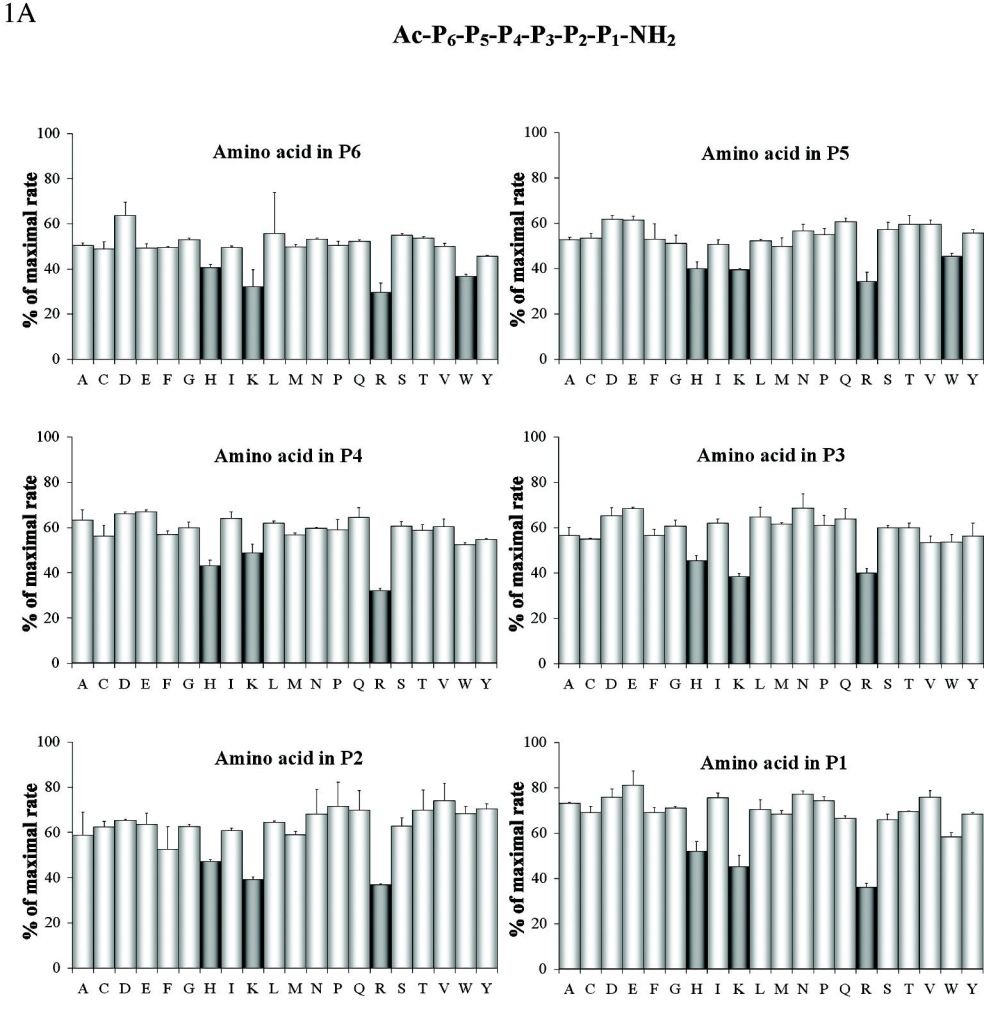
A

Poly-(L)-arginine	**PC5/6**	**PC7**	**Furin**	**PACE4**
		***K*i (nM)**		
Tetra-(L)-arginine	2500	6000	6400	>10000
Penta-(L)-arginine	450	1100	990	980
Hexa-(L)-arginine	430	1050	114	520
Hepta-(L)-arginine	575	312	68	240
Octa-(L)-arginine	450	200	61	150
Nona-(L)-arginine	150	120	42	110

B

Poly-(D)-arginine	**PC5/6**	**PC7**	**Furin**	**PACE4**
		***K*i (nM)**		
Hexa-(D)-arginine	265	1875	106	580
Nona-(D)-arginine	19	81	1.3	-

1A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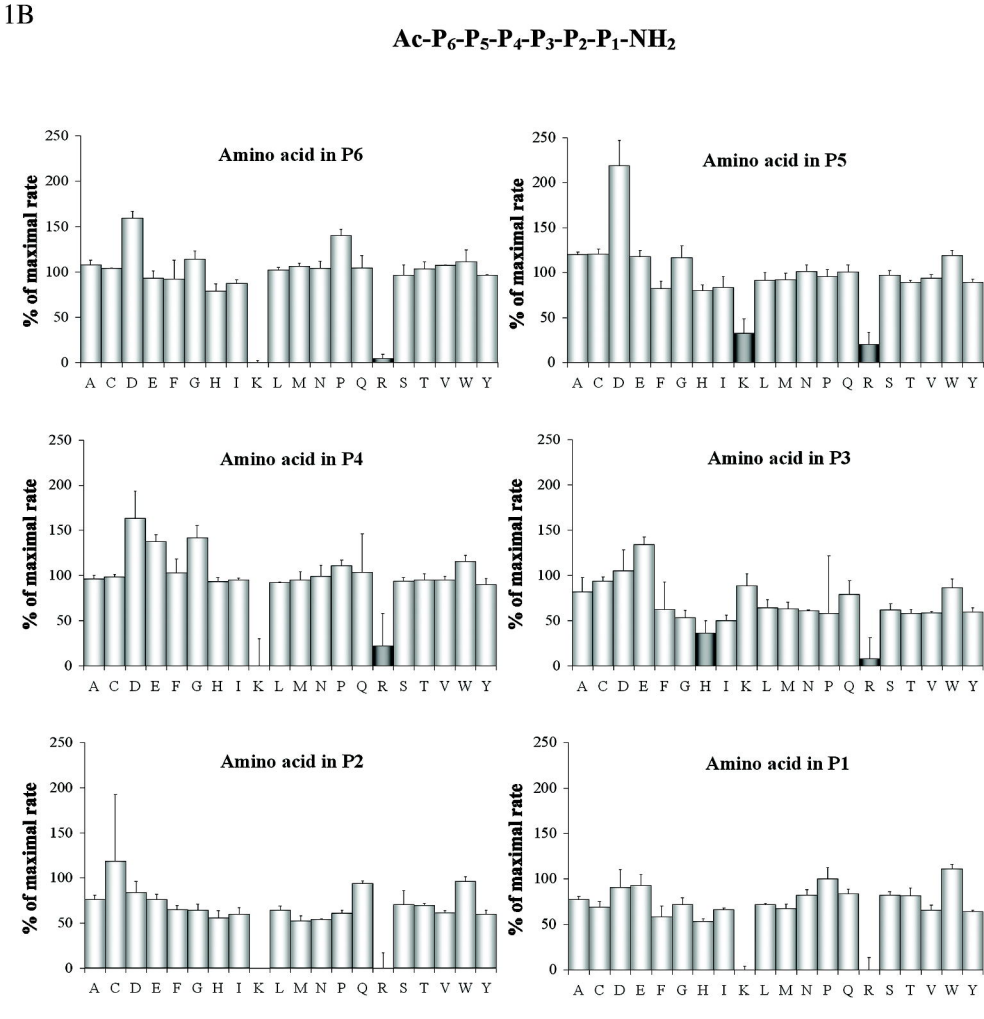
Amino acid in P6
Amino acid in P5
Amino acid in P4
Amino acid in P3
Amino acid in P2
Amino acid in P1
% of maximal rate
0
20
40
60
80
100
A C D E F G H I K L M N P Q R S T V W Y

1B


Ac-P6-P5-P4-P3-P2-P1-NH2
Amino acid in P6
Amino acid in P5
Amino acid in P4
Amino acid in P3
Amino acid in P2
Amino acid in P1
% of maximal rate
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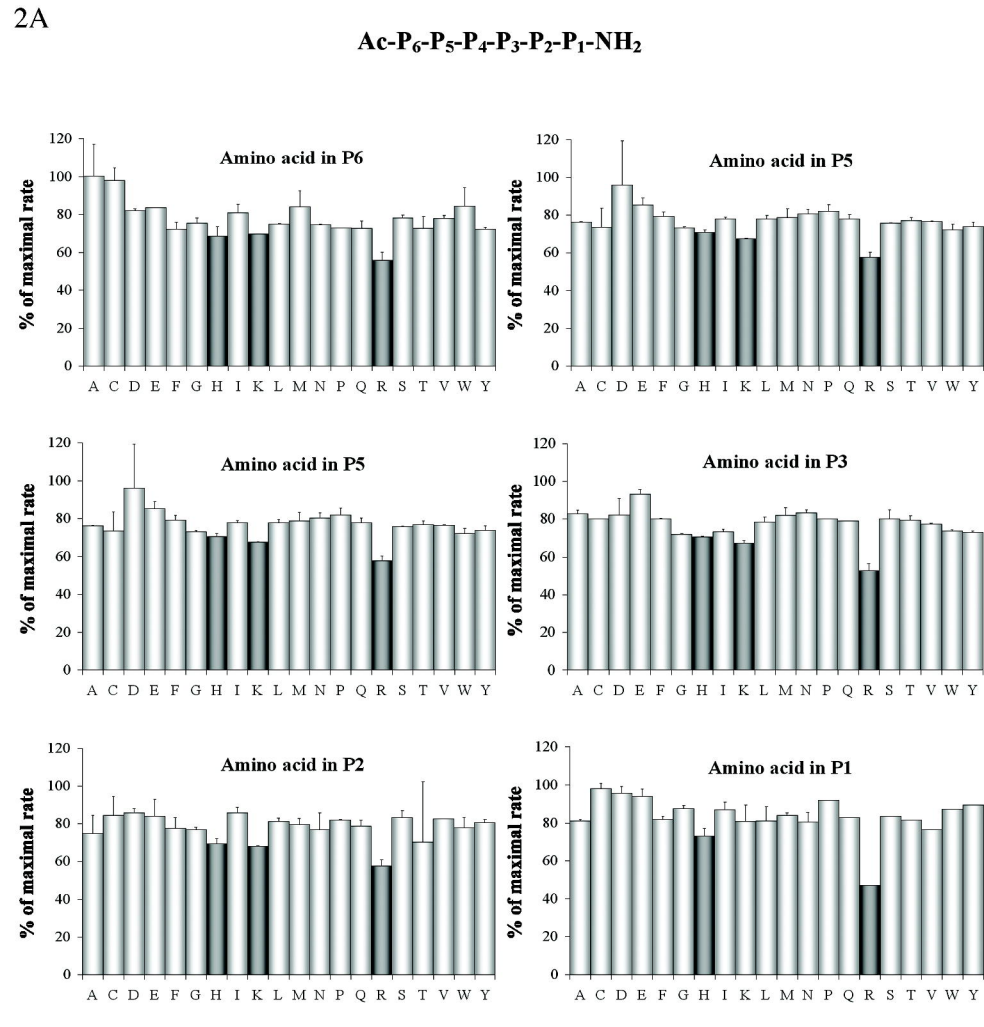
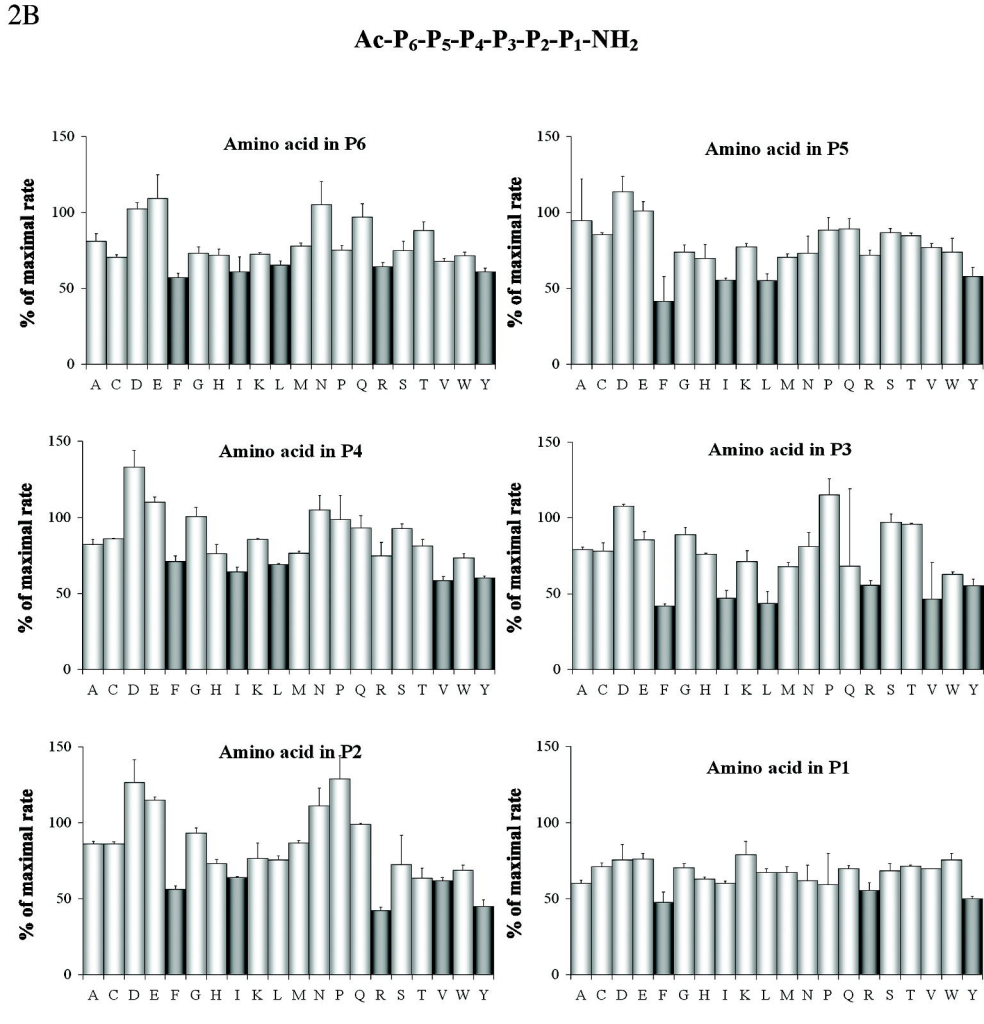
50
100
150
200
250
A C D E F G H I K L M N P Q R S T V W Y

2A

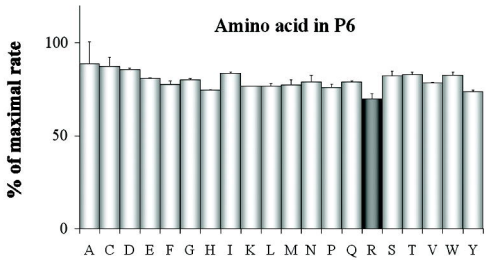

Ac-P6-P5-P4-P3-P2-P1-NH2
Amino acid in P6
Amino acid in P5
Amino acid in P5
Amino acid in P3
Amino acid in P2
Amino acid in P1
% of maximal rate
0
20
40
60
80
100
120
A C D E F G H I K L M N P Q R S T V W Y

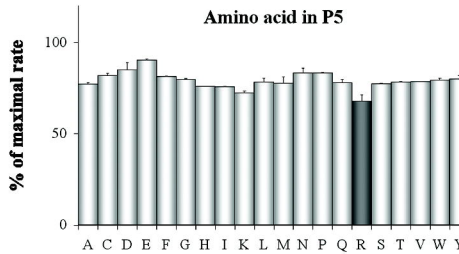
2B

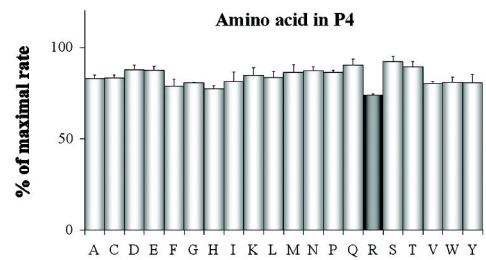

Ac-P6-P5-P4-P3-P2-P1-NH2
Amino acid in P6
Amino acid in P5
Amino acid in P4
Amino acid in P3
Amino acid in P2
Amino acid in P1
% of maximal rate
150
100
50
0
A C D E F G H I K L M N P Q R S T V W Y

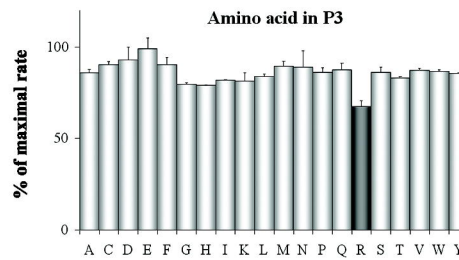
3

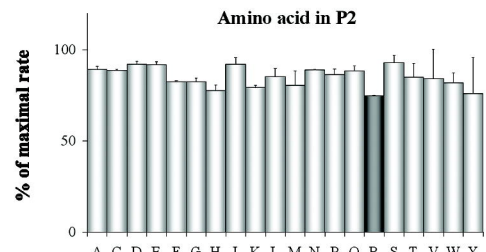
Ac-P_6-P_5-P_4-P_3-P_2-P_1-NH_2

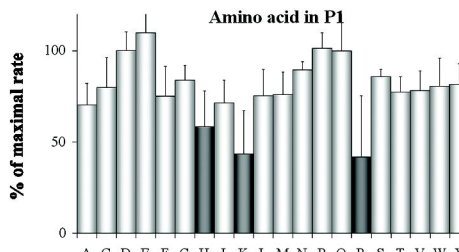




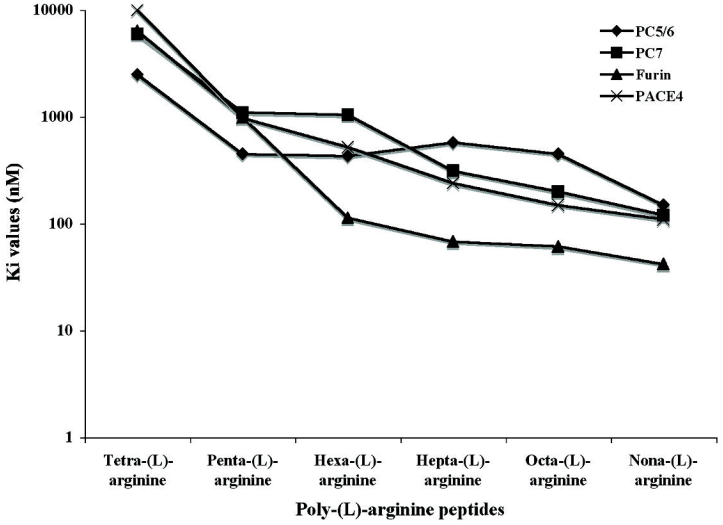




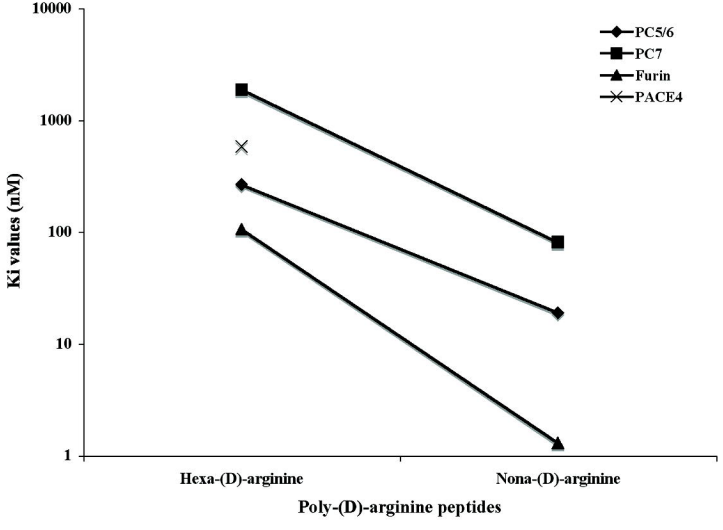




4A


10000
1000
100
10
1
Ki values (nM)
PC5/6
PC7
Furin
PACE4
Tetra-(L)-arginine
Penta-(L)-arginine
Hexa-(L)-arginine
Hepta-(L)-arginine
Octa-(L)-arginine
Nona-(L)-arginine
Poly-(L)-arginine peptides

4B


10000
1000
100
10
1
Ki values (nM)
PC5/6
PC7
Furin
PACE4
Hexa-(D)-arginine
Nona-(D)-arginine
Poly-(D)-arginine peptides