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Inhibitor screening of proprotein convertases using positional scanning libraries

Iris Lindberg and

University of Maryland-Baltimore ilind001@umaryland.edu

Jon R. Appel

Torrey Pines Institute for Molecular Studies

Summary

Proprotein convertases represent an important class of biosynthetic enzymes that are increasingly viewed as targets for therapeutic approaches to infection, cancer and potentially endocrine disorders. The identification of potent inhibitors can be accomplished by screening synthetic combinatorial libraries containing thousands of small molecules to millions of peptides. In this chapter, the screening of positional scanning libraries is described for the identification of PC1/3 and PC2 inhibitors.

Keywords

prohormone convertase 1/3 (PC1/3, formerly known as PC1); prohormone convertase 2 (PC2); enzyme inhibitor; synthetic combinatorial libraries; positional scanning; mixture-based libraries

1. Introduction

The proprotein convertases, discovered over 20 years ago, represent a family of eukaryotic subtilases with restricted tryptic-like specificity. These enzymes are involved in a wide variety of physiological processes, from endocrine control to synthesis of growth factors and neuropeptides. Proprotein convertases are typically divided into two classes, those with restricted tissue expression, such as the neuroendocrine enzymes prohormone convertases 1/3 and 2, and those with wide expression, such as furin, responsible for the proteolytic maturation of a large number of circulating molecules. Furin is also involved in pathogenic processes such as viral and bacterial infection, where pathogenic organisms take advantage of host processing machinery, and in the development of oncogenic processes due to the need for furin in metastatic events. The development of furin inhibitors is a rapidly moving field, and a variety of different inhibitors ranging from peptides to small molecules have been described (reviewed in (1). While a potent, cell-permeable, small molecule proprotein convertase inhibitor has not yet been achieved, much progress has recently been made towards this goal (2); (3); (4); (5). Development of specific prohormone convertase inhibitors is also expected to yield therapeutically useful molecules (6). For example, blocking the synthesis of glucagon- a PC2-specific event- could result in better glycemic control, whereas blocking the synthesis of ACTH, largely under the control of PC1/3, could be helpful in pituitary disease. As described below, we have taken a combinatorial library approach to the identification of convertase inhibitors,

Combinatorial library synthesis and screening methods, which enable the rapid identification of highly active compounds, have revolutionized basic research and drug discovery. A number of different combinatorial approaches based on the principles of solid phase synthesis have been used to generate enormous molecular diversities, including peptides, peptidomimetics, small organic molecules, and heterocyclic compounds (7); (8); (9); (10). The main advantage of the various combinatorial approaches compared to traditional drug synthesis and screening is the fact that very large numbers of compounds can be simultaneously synthesized and rapidly screened in biological assays.

Positional scanning synthetic combinatorial libraries are composed of positional libraries or sublibraries, in which each diversity position is defined with a single building block, while the remaining positions are composed of mixtures of building blocks (11). Each positional sublibrary represents the same collection of individual compounds. Further, mixture-based libraries, when arranged in a positional scanning format, provide extensive structure-activity information in any given assay. As a simple means to illustrate the positional scanning library concept, a tripeptide combinatorial library is illustrated in Figure 1. Three different amino acids are incorporated at each of the three diversity positions, resulting in 27 (3^3) individual peptides. When the same peptides are arranged as a positional scanning library, only 9 peptide mixtures ($3 \text{ amino acids} \times 3 \text{ positions}$) need to be synthesized. Each of the three positional sublibraries, OXX, XOX, and XXO, contains the same diversity of peptides, but differ only in the location of the position defined with a single amino acid. The O positions represent one of the four amino acids while the remaining two positions are mixtures (X) of the same four amino acids. Shown below each mixture are the 9 peptides (3^2) that make up that mixture. In this example, assume that alanine-arginine-threonine, or ART is the only tripeptide in this library that is recognized by a given receptor. Since each positional sublibrary contains the same diversity of peptides, the ART tripeptide (outlined below in each sublibrary in Figure 1) is present in all three positional sublibraries. Thus, the only mixtures with activity are AXX, XRX, and XXT -because the ART tripeptide is present only in those mixtures. The combination of these amino acids in their respective positions yields the tripeptide ART, which would then be synthesized and tested for its activity against this receptor. It should be noted that the activity observed for each of the three mixtures (AXX, XRX, and XXT) is due to the presence of the tripeptide ART within each mixture, and not due to the individual amino acids (A, R, and T) that occupy the defined positions. In more complex libraries, more than one mixture is often found to exert activity at each position. Selection of the building blocks for the synthesis of individual compounds is based first on activity and then on differences in the chemical character of the building block.

Although the above example is a simple representation of the arrangement and use of a positional scanning library, the concepts described here apply to all types of mixture-based libraries having defined and mixture positions, using either amino acids (D or L), or scaffolds bearing various chemical substituents at different positions. For example, a hexapeptide library using 20 amino acids represents a total of 4.9×10^7 (20×19^5 ; cysteine is omitted in the mixture positions) individual peptides (Figure 2). This can be formatted into a positional scanning library of 120 mixtures ($20 \text{ amino acids} \times 6 \text{ positions}$). This library will be used to describe our identification of a precise hexapeptide inhibitor of PC1/3 (12), although similar principles apply to the identification of a nonpeptide inhibitor of PC2 (6).

2. Materials

1. Positional scanning libraries are available on a collaborative basis from the Torrey Pines Institute for Molecular Studies. The synthesis of various peptide and nonpeptide libraries has been described (13) (7). In the following protocol a

hexapeptide positional scanning library of 120 mixtures is used. Concentrations used range from 1 to 5 mg/ml in water, and the library can be stored at 4C for several weeks while in use or -20 C when not in use.

2. Purified recombinant PC enzymes are prepared from the conditioned medium of overexpressing CHO cells (14); (15). ProPC2 is obtained as a zymogen and should be activated by dilution in reaction buffer prior to use. PC1/3, obtained as an active enzyme, is prone to autocatalytic conversion to smaller enzyme products which may have differential susceptibility to inhibitors. Intermediate stock solutions should be prepared in reaction buffer (it must contain detergent and BSA to avoid losses by adsorption), stored on ice, and used within hours; stock solutions of both enzymes are relatively stable to three cycles of freezing and thawing.
3. The fluorogenic substrate used is pERTKR-aminomethylcoumarin, obtained from Peptides International, Lexington, KY. Stock is made up as 20 mM solution of peptide in DMSO; net peptide weight is usually 75% (the remainder being salts and water).
4. 96-well polypropylene round-bottomed microtiter plates (Costar 3365).
5. Multichannel pipettors capable of delivering 5- 40 ul in octuplicate and plastic reservoirs to use with these pipettors.
6. The PC2 assay buffer contains 100 mM sodium acetate, pH 5.0, 2 mM CaCl₂, 0.2 % n-octylglucoside (RPI), 0.1% NaN₃, and 0.1 mg/ml crystalline Fraction V BSA (Roche). For PC1/3 the assay pH is adjusted to 5.5.
7. A fluorometer plate reader, either filter or monochromator-based, with the ability to perform kinetic reads (accumulation of product over time). We read our plates from the top since our plates are opaque.

3. Methods

3.1 General considerations

Screening many plates is an operation that must be carefully timed, as most fluorometers will only read a single plate at a time. We stagger the start of the enzyme assays such that we can obtain 25 minute kinetic reads of each new plate every 30 minutes. Depending on the speed of the equipment and operator, this timing may need to be varied.

We use polypropylene round-bottom plates for two reasons. One, the round-bottomed format cuts the amount of reactants needed in half vs a flat-bottomed plate (50 vs 100 ul). Secondly, convertases are extremely sticky, and proteins tend to stick less tightly to polypropylene than to polystyrene. However, flat-bottomed plates will certainly work.

3.2. Experiment preparation

1. Enzyme Preparation: Prepare sufficient enzyme (diluted in reaction buffer, 40 ul per well) for the number of plates to be assayed within the next 2-3 hours. Depending on the sensitivity of the instrument to be used, pg to ng/well concentrations are used for PC2; PC1 reactions will require 20-50x as much enzyme due to its lower specific activity. For maximum sensitivity in inhibitor detection, the amount of enzyme used should be kept as low as possible while still generating adequate reproducibility and signal-to-noise ratio for the uninhibited control. Remember to prepare sufficient enzyme such that the bottom of the multichannel pipettor reservoir is covered (about 1 ml extra). Example: for four 96

well plates which will require 40 μ l of diluted enzyme, prepare $0.04 \mu\text{l} \times 100 \text{ wells} \times 4 \text{ plates plus } 1 \text{ ml} = 17 \text{ ml}$.

2. Substrate preparation: Dilute the standard substrate, pERTKR-aminomethylcoumarin to 0.5 mM in water. Use 5-10 μ l per well (0.05 to 0.1 mM final concentration). Again, sufficient substrate must be prepared to accommodate the reservoir. It is possible to use substrate amounts lower than this (substrate is the most costly ingredient in the screen), which may increase sensitivity to competitive inhibitors, but the reproducibility and signal-to-noise ratio must first be checked.
3. Library preparation: Most positional scanning libraries are supplied in high concentrations (1-10 mg/ml), dissolved either in water, formamide, or DMSO. Peptide libraries containing hydrophobic amino acids in the defined positions are particularly likely to have various amounts of insoluble material. It is extremely important that the tubes be well mixed prior to pipetting (see below).

3.3 Enzyme inhibition assay

1. Using an 8-channel pipettor (see Note 1), pipet 40 μ l of enzyme solution into all wells of a 96 well plate on a bed of wet ice except the end columns (these will become background controls).
2. Pipette 5 μ l of well-mixed inhibitor (i.e. library) solution to duplicate rows starting from the second row. Pipet vehicle (the solution the library arrives in) into the control rows. Remember to mix tubes well prior to each row of pipetting, as the inhibitor mixtures often consist of particulate suspensions. We accomplish mixing using a dedicated plate shaker, a GlasCol Pulse-Vortexer Mixer, and shake the plate such that a homogeneous suspension is achieved prior to pipetting each row of samples. The presence of a homogeneous suspension should be verified by visual inspection prior to pipetting; samples settle remarkably quickly.
3. Preincubate plate for 30 minutes at room temperature to permit the inhibitor to bind the enzyme; note the start time (see Note 2). Preheat the fluorometer by running a mock plate.
4. Start the reaction by adding 5 μ l of diluted substrate. There should be no bubbles visible. If this is a problem, briefly spin down the plates to break the bubbles. Note the time the reaction was started.
5. Place the plate in the fluorometer chamber and measure fluorescence at timed intervals. The fluorometer (380 excitation, 480 emission) should be pre-warmed and programmed to take 2-3 multiple reads over each minute, reading each plate once a minute repetitively for at least 25 minutes. After an initial period of warming, the rate of release of the fluorescent product aminomethylcoumarin will be directly proportional to the rate of the reaction. The rate of hydrolysis of inhibited wells (relative to control samples lacking inhibitors) is often- though not always- linear and can be used as a direct measure of the relative inhibition by each library mixture. It is helpful to inspect the progress curves during the reaction so that you can see whether the inhibited rates are linear and exhibit a lower slope than the non-inhibited rates (indicative of competitive inhibition); or are non-linear (this can indicate noncompetitive inhibition). This progress curve feature is available on most kinetic fluorometers. If rates are linear, calculate the average rates for each well, and print out both the curves and the rates for each plate (or copy to a spreadsheet for later entry into a

¹A multi-channel pipette (or a robotic pipetting station) is essential for pipetting libraries onto microtiter plates; this minimizes pipetting errors and improves assay reproducibility.

²Some inhibitors display time-dependent inhibition; for these compounds, a much better signal will be observed using longer preincubation periods

graphics program). If inhibited rates are not linear, use the maximum rate for each well as a measure of its inhibited velocity, but make sure an average of at least 9 time points are averaged to get this maximum (see Note 3), and note the time of preincubation as well as of the assay as both of these parameters will impact the maximum rate. In either case, duplicates should agree to within 15%; if they do not, adjust assay parameters until this precision level is obtained.

8. Immediately after beginning the first read, pipet enzyme and inhibitor into the second plate for the second preincubation. Note the time. Add substrate after 30 minutes at room temperature, at which time the first plate should be finished with data collection and the second plate can be inserted into the fluorometer. Immediately pipet the third plate and continue until all of the data have been collected.

3.4. Optimizing and confirming inhibition

A good understanding of the various assay parameters, such as signal-to-noise ratio, variability, and sensitivity is required for the successful identification of active compounds from the library. The most important parameter to control for an assay system is the variability. To optimize assays one can vary preincubation time with inhibitor; enzyme, substrate and inhibitor concentrations; and salt concentration in the buffer- salt, for example, can radically affect the potency of charged inhibitors. The use of repeated experiments and averaged data ensures the accurate identification of individual compounds having significant activity. For inhibitor variation, depending on the amount of inhibition originally observed, use half to 1/20th of the amount of inhibitor originally pipetted; dilutions (in water) can be prepared in parallel plates (remember to mix the original library tubes well and pipet as a homogeneous suspension!). Our experience indicates that it is unlikely that dilutions greater than 1/10 will exhibit much inhibition (see Note 4).

3.5 Positional scanning library deconvolution

In order to identify the most active individual compounds from the positional scanning library, the individual peptides that correspond to the combination of the amino acids defined in the most active mixtures at each position are synthesized and tested. For practical purposes, the number of amino acids selected from each position that will be used to synthesize the individual peptides should be minimized as the number of compounds to be made rises exponentially (see Note 5). For example, if two amino acids were selected from each position of a hexapeptide library, one would need to synthesize 64 peptides (2⁶).

Successful deconvolution of active individual compounds from mixture-based libraries is dependent on reproducible screening data and clear dose-response activities of the most active mixtures. In most cases, dose-response curves can be determined for the most active mixtures, and activities based on calculated IC₅₀ values are used to select the building

³Data analysis: It is extremely important to have a good non-inhibited control value since all other values depend on this value. To obtain this, use a sufficient number of wells (usually between 3 to 5), which achieves less than 10% variability. For final data reduction, subtract the no-enzyme background from all wells, average the non-inhibited controls, and plot experimental data as the percent inhibition (not activity) and as a bar graph with either amino acid or tube number as the X axis. Any convenient graphics program can be used; we use Prism. Some mixtures may stimulate; this may not be an artifact, and these data should also be collected.

⁴Library and enzyme concentration should be adjusted in order to perform the assay at the optimal sensitivity to detect inhibition by mixtures. For example, if there is no inhibition from the library, repeat the screening at a higher library concentration. Conversely, if high inhibition (>80%) is observed for all the mixtures of the library, repeat the screening at a lower library concentration. A two- to four-fold change in library concentration should result in a differentiating screening profile, in which there are mixtures that still inhibit as well as mixtures with little activity.

⁵Often building blocks of similar chemical character will yield similar activities at a given position. This may indicate that a number of analogs of the same compound are responsible for the observed activity. Similar building blocks can be excluded from selection to reduce the number of final compounds needed to be synthesized. However, one can later synthesize analogs of the most active individual compounds using the building blocks that were originally excluded.

blocks that will be included in the synthesis of individual compounds (see Note 6). Some positions may exhibit more distinct residue preferences than others; this is due to binding requirements. For example, the P3 position is not nearly as discriminating as the P1 position for PC1/3 (12).

Upon synthesis and testing of individual peptides derived from the screening of positional scanning libraries, one should be able to confirm the activity of the selected mixtures. It is important to note that it is the activity of the individual peptides within a mixture, and not the amino acid in the defined position of the mixture, that results in the observed inhibitory activity. If no inhibitory activity is found within the set of individual peptides, then either additional peptides need to be prepared that include amino acids that were not initially selected, or an iteration can be prepared using the most active mixture as a starting point. Upon iterating an active mixture and defining additional positions of the library with specific amino acids, the resulting mixtures become progressively less complex since they contain fewer different peptides, and this should result in an increase in inhibitory activity relative to the original mixtures from the library.

A representative screen of the hexapeptide positional scanning library with PC1/3 is shown in Figure 3. To identify the hexapeptides from this library that inhibit PC1/3, the amino acids defined in the most active mixtures at each position were selected to make a set of individual peptides. In this example, twelve hexapeptides were made based on the combination from selecting leucine in position 1; lysine, leucine, methionine and tyrosine in position 2; arginine in position 3; histidine, threonine, and valine at position 4; and lysine and arginine at positions 5 and 6, respectively. The activities of these peptides are shown in Figure 4, in which Ac-LLRVKR-NH₂ was found to be the most active inhibitor of PC1/3 (12). Two years later, this exact hexapeptide was found within a natural inhibitor of this enzyme (16).

A strategy termed biometrical analysis has been developed to systematically compare the results obtained from screening a peptide library composed of millions of sequences with the millions of sequences within protein databases. This approach is based on the assumption that each amino acid in a peptide epitope or ligand in other protein/protein interaction contributes independently and additively to recognition or, in general terms, strength of interaction. Hence, the stimulatory value of each amino acid in a given position can be added to that of the amino acid in the next position throughout the length of the peptide. Consequently, a stimulatory score can be calculated for each peptide. A scoring matrix is generated by transforming the screening data of each of the 20 amino acids defined in each position of the library. Individual peptides are given a score calculated by adding the individual activities of the amino acids for the length of the library. This matrix is then used to score all the overlapping peptides of a given length in the protein databases and thus identify the sequences with the highest score. From this list a number of sequences with the highest scores can be synthesized and tested to confirm the activities (17), (18). We have used the biometrical analysis for T cell clones of known and unknown specificities. When T cell ligands are known, they are found within the set of peptides with the highest scores. This approach has also been successfully used for the identification of T cell ligands for a CD4⁺ T cell clone from a patient with Lyme disease. Both microbial epitopes from proteins in *Borrelia burgdorferi* (the infectious bacteria of Lyme disease) and candidate autoantigens

⁶In a number of examples of library screening data, the distinction between active and inactive mixtures is more difficult to determine, because either the specificities of the most active mixtures are not very clear, or the signal-to-noise ratio of the assay is less than three-fold. In these cases, dose-response determinations may not be possible. Another strategy that can be useful is to compare the activity of a given mixture relative to the average mixture activity at that diversity position. The data analysis of complex mixtures is no different from the data obtained using individual compounds. One simply follows the activity that is significantly affecting the assay. In other words, distinguishing between active and inactive samples is independent of the complexity of these samples.

were identified (unpublished results). These data further support the idea that combinatorial screening methods, combined with novel algorithms, have the potential to identify naturally-occurring molecules.

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Tripeptide library

Position 1	O X X	Position 2	X O X	Position 3	X X O
1 A X X		4 X A X		7 X X A	
2 R X X		5 X R X		8 X X R	
3 T X X		6 X T X		9 X X T	
A X X R X X T X X		X A X X R X X T X X		X X A X X R X X T	
A A A R A A T A A		A A A A R A A T A		A A A A A R A A T	
A A R R A R T A R		A A R A R R A T R		A R A A R R A A T	
A A T R A T T A T		A A T A R T A T T		A T A A T R A A T	
A R A R R A T R A		R A A R R A R T A		R A A R A R A T A	
A R R R R R T R R		R A R R R R R T R		R R A R R R R R T	
A R T R R T T R T		R A T R R T R T T		R T A R T R R T T	
A T A R T A T T A		T A A T R A T T A		T A A T A R T A T	
A T R R T R T T R		T A R T R R T T R		T R A T R R T R T	
A T T R T T T T T		T A T T R T T T T		T T A T T R T T T	

Figure 1. Conceptual illustration of a tripeptide positional scanning library
 O, defined functionality ; X, mixture of functionalities.

Hexapeptide library									
Position 1	O	X	X	X	X	X	X	→ 20 Mixtures	1. AXXXXX ↓ 20. YXXXXX
Position 2	X	O	X	X	X	X	X	"	
Position 3	X	X	O	X	X	X	X	"	
Position 4	X	X	X	O	X	X	X	"	
Position 5	X	X	X	X	O	X	X	"	
Position 6	X	X	X	X	X	O	X	"	
Total # mixtures							120 Mixtures		
Total # peptides							$20 \times 19^5 = 4.9 \times 10^7$		

Figure 2. Representation of hexapeptide positional scanning library

O, one of 20 L-amino acids in defined position; X, equimolar mixture of 19 L-amino acids (cysteine omitted).

Figure 3.
Inhibition of PC1/3 activity by a hexapeptide positional scanning library (final concentration 1 mg/ml).

Position	1	2	3	4	5	6
Amino acid	L	K,L,M,Y	R	H,T,V	K	R

		PC1/3	
		% Inhibition at	PC1/3 Ki
		100 ng/ml	(nM)
Peptide			
1	Ac-LKRHKR-NH ₂	11	>100
2	Ac-LKRTKR-NH ₂	19	>100
3	Ac-LKRVKR-NH ₂	59	5.7
4	Ac-LLRHKR-NH ₂	18	60
5	Ac-LLRTKR-NH ₂	34	16
6	Ac-LLRVKR-NH ₂	75	3.2
7	Ac-LMRHKR-NH ₂	10	>100
8	Ac-LMRTKR-NH ₂	25	>100
9	Ac-LMRVKR-NH ₂	61	4.9
10	Ac-LYRHKR-NH ₂	5	>100
11	Ac-LYRTKR-NH ₂	22	>100
12	Ac-LYRVKR-NH ₂	51	6.5

Figure 4.

Amino acids selected to synthesize individual peptides and the inhibitory activities of the hexapeptides against PC1/3.