Fragment-Based Drug Discovery: Comparing Labeled and Label-Free Screening of β-Amyloid Secretase (BACE-1) Using Fluorescence Spectroscopy and Ultrafast SPE/MS/MS

Application Note

Authors

Lauren E. Frick and William A. LaMarr
Agilent Technologies, Inc.
Wakefield, MA, USA

Introduction

Fragment-based screening offers advantages over traditional high-throughput screening by allowing more comprehensive coverage of chemical space, but the typical low potency of fragments leads to the frequent use of physical methods that detect binding. The few existing activity-based biochemical assays tend to employ optical methods, such as fluorescence spectroscopy (FS), which can be subject to confounding factors due to the high concentrations of compound needed to detect activity. Here we screen β-amyloid secretase (BACE-1) against a fragment library using two substrates, a labeled and an unlabeled peptide, which were detected either by FS or ultrafast SPE/MS/MS using the Agilent RapidFire High-throughput Mass Spectrometry (MS) System. Different kinetic parameters, hit rates, and hit sets were obtained depending on the substrate and detection method, suggesting that using fluorescent labels and optical detection methods can lead to follow-up of compounds that are inactive against the unlabeled, more biologically relevant substrate. RapidFire-MS, which allows the direct study of native molecules, eliminates these potential pitfalls.

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Experimental

Chemicals and reagents

Fluorescently-labeled or unlabeled BACE-1 substrate and product peptide standards were of the sequences Mca-SEVNLDAEFR-K(Dnp)-RR, Mca-SEVNL, DAEFR-K(Dnp)-RR, SEVNLDAEFR, SEVNL, and DAEFR. The unlabeled substrate peptide was purchased from Sigma-Aldrich, St. Louis, MO. The labeled substrate peptide and BACE-1 enzyme were purchased from R&D Systems, Inc., Minneapolis, MN. Standard peptides representing the cleavage products of both peptides were synthesized by American Peptide Company, Sunnyvale, CA. The reference inhibitor was purchased from EMD Biosciences, Inc., San Diego, CA. The fragment library was a 1,000-compound diversity subset of the Maybridge Ro3 Fragment Library, purchased from Thermo Fisher Scientific, Waltham, MA.

Sample preparation

BACE-1 reactions in a 50 µL volume were run using the following final conditions: 50 mM NaOAc pH 4.5, 50 mM NaCl, 0.03 % BSA, 0.0025 % Genapol, and 20 nM BACE-1 enzyme. Screening reactions were run with the following additions: 1 mM test compound, 2 % DMSO vehicle as an uninhibited control, and 1 µM β-secretase Inhibitor IV in 2 % DMSO as a fully-inhibited control. Labeled reactions contained 10 µM substrate and were run at room temperature for 120 minutes. Unlabeled reactions contained 2 µM substrate and were run at room temperature for 180 minutes. Both types of reactions were quenched with 10 µL 10 % formic acid containing 2 µM of the opposite product standard as an internal standard (that is, labeled product standard in the unlabeled substrate reaction and *vice versa*).

Post-quench, 45 µL ddH_2O was added to fluorescent reactions to increase the reaction volume height, allowing for sensitive and consistent data collection in the fluorescence spectrophotometer.

RapidFire triple quadrupole conditions

A RapidFire 360 High-throughput MS System and RapidFire integrator software were used for the analysis. Samples were analyzed at a rate of approximately 10 seconds per sample using the conditions shown in Table 1.

Fluorescence spectroscopy parameters

Samples were analyzed at a rate of approximately 2 seconds per sample using the conditions shown in Table 2. Wavelengths were optimized and data were collected for the Mca-SEVNL product peptide.

Table 1. RapidFire LC/MS conditions.

RapidFire conditions		
Buffer A	Water with 0.1 % formic acid; 1.5 mL/min flow rate	
Buffer B	100 % acetonitrile with 0.09 % formic acid and 0.01 % trifluoroacetic acid; 1.25 mL/min flow rate	
Injection volume	10 µL	
SPE cartridge	Agilent RapidFire cartridge A (reversed-phase C_4 chemistry, G9203A)	
RF state 1	sip sensor	
RF state 2	3,500 ms	
RF state 3	5,000 ms	
RF state 4	500 ms	
MRM transitions	**Q1**	**Q3**
Labeled substrate	668.0	101.8
Labeled product	777.3	532.1
Unlabeled substrate	590.5	216.8
Unlabeled product	561.3	217.0

Table 2. Fluorescence spectrometry conditions.

Cary Eclipse Fluorescence Spectrophotometer conditions	
Data mode	Fluorescence
Excitation wavelength	394 nm
Emission wavelength	326 nm
Excitation slit	5 nm
Emission slit	5 nm
Average time	0.1 s

Data analysis

Cary Eclipse Advanced Reads software was used to acquire fluorescence data. RapidFire Integrator v3.6 software was used for MS peak integration. Microsoft Excel 2007 and GraphPad Prism 5 were used for data analysis and calculation of kinetic parameters. Hits were defined as fragments that produced normalized product signal less than three standard deviations below the average of the values obtained for the eight DMSO-only control wells on each plate. Similarly, autofluorescence was defined as unnormalized product signal greater than three standard deviations above the uninhibited average for each plate.

Results and Discussion

Assay development

Functional biochemical BACE-1 assays were optimized around each substrate, with full characterization of buffer requirements, enzyme linearity, binding kinetics, DMSO tolerance, and inhibition by a reference compound (β-secretase Inhibitor IV). While the assays displayed similar linearity at room temperature, the BACE-1 enzyme exhibited very different affinities for the two different substrates (Figure 1, left panels). A standard K_m curve could be generated for the unlabeled peptide (calculated K_m of 22.4 µM), but curves could not be constructed for the labeled peptide, presumably due to poor substrate solubility at the higher concentrations required. These data suggest that the labeled peptide is a significantly less efficient substrate for the enzyme, which could alter the assay results.

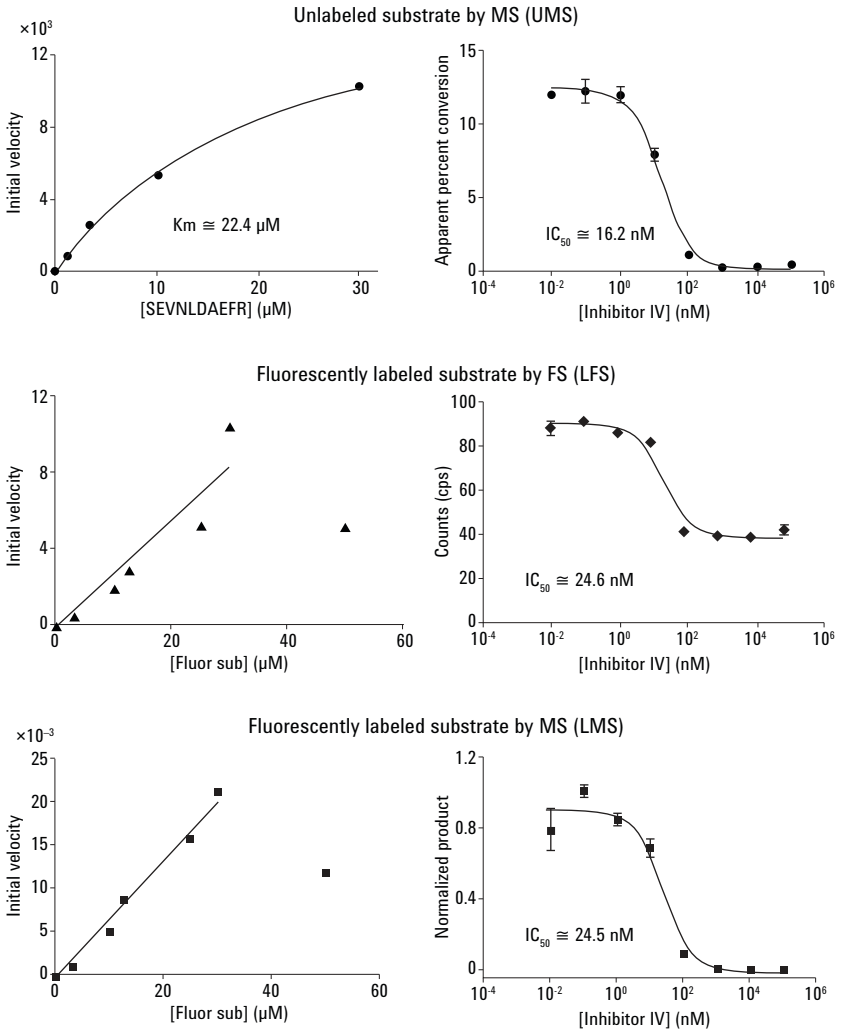

Figure 1. Kinetic parameters of different substrates by mass spectrometry (MS) and fluorescent spectroscopy (FS): unlabeled substrate by MS (UMS), fluorescently-labeled substrate by FS (LFS), and fluorescently-labeled substrate by MS (LMS).

Reference inhibition curves with Inhibitor IV, however, produced similar values of 16.2 nM for the unlabeled substrate and 24.6 nM and 24.5 nM for the labeled substrate by FS and MS, respectively (Figure 1, right panels). These values agreed quite well, both with each other and with the given literature value of 15 nM.[1] Z′ values comparing DMSO-only wells with wells containing 1 µM inhibitor IV were between 0.61 and 0.71 for all assays, with n=12-24.

Fragment library screening

After robust assays were developed, each substrate was employed in a screen of BACE-1 against a 1,000-compound diversity subset of the Maybridge Ro3 Fragment Library. Compounds were screened in 96-well plate format at a final concentration of 1 mM. Initial screening of a fragment library generated different hits and hit rates among the various assay formats (Figure 2). Compounds of interest (primarily those registering as hits in certain assays but not others) were chosen for confirmation screening. Follow-up studies of these selected hits revealed the presence of several classes of compounds with differing inhibitory characteristics towards the BACE-1 reaction.

Hits observed by MS only

Follow-up of selected hits confirmed that compound autofluorescence (AF) obscured several hits in the FS data, including the most potent analyte. Titration of that compound revealed a concentration-dependent increase in signal in the FS assay, suggesting AF, while the MS data were consistent with a traditional inhibition curve (Figure 3).

	UMS	LMS	LFS	All 3
UMS	211	14	41	-
LMS	14	32	22	-
LFS	41	22	122	-
All 3	-	-	-	8

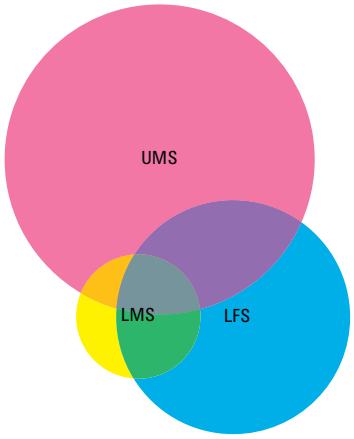

Figure 2. Table and Venn diagram of initial screening results displaying different hit rates and hit sets by assay format: unlabeled substrate by MS (UMS), labeled substrate by MS (LMS), and fluorescently-labeled substrate by FS (LFS).

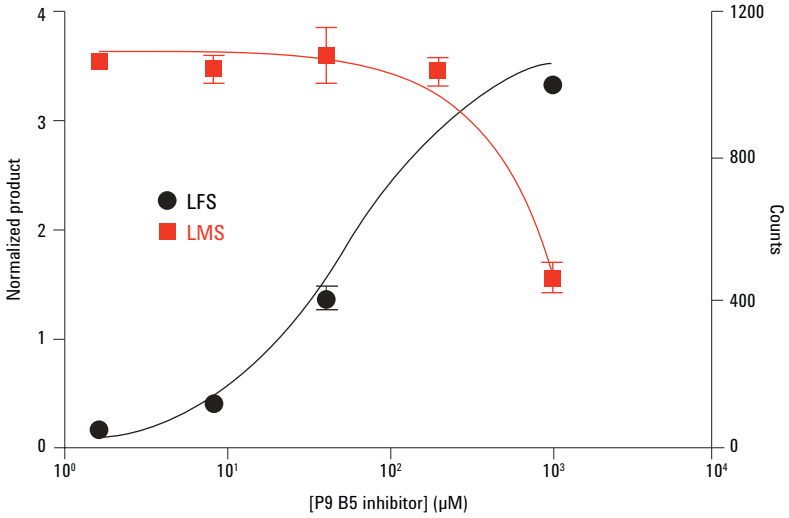

Figure 3. Inhibition observed by MS appears as concentration-dependent increase in signal by LFS.

Hits observed with the unlabeled peptide only

A second class of inhibitors was detected in the unlabeled assay (UMS) whose members were not found with the fluorescent peptide (LFS or LMS). Because MS eliminates the need for unnatural modification of substrates, it allows the study of more biologically relevant molecules. These more realistic substrates could reveal activities that are lost with modified peptides, possibly due to altered binding, as in this case was clearly revealed by the K_m experiments.

Hits observed with the labeled peptide only

Yet another set of compounds was uncovered consisting of those molecules that appear as hits when the labeled peptide is employed (as in the LFS and LMS assays), but do not show significant inhibition when the more native substrate is used (UMS, Figure 4). These results suggest that compounds may exist that interfere with the enzyme's ability to bind the peptide carrying the bulky label but not with the tighter binding exhibited by the enzyme for the unlabeled substrate, raising the possibility of misleading data being produced when modified substrates are employed.

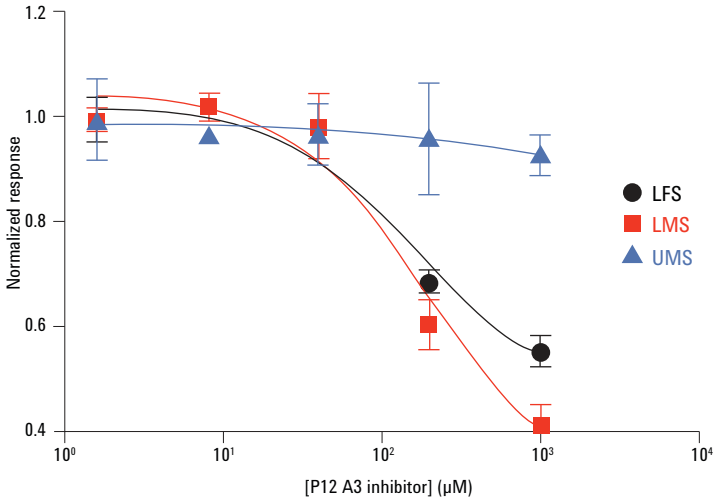

Figure 4. Inhibition observed with the labeled peptide is not seen with the unlabeled peptide.

Conclusions

Robust functional biochemical assays were developed for both a labeled and an unlabeled substrate of the BACE-1 enzyme, with data collection by both MS and FS. Using these assays to screen a fragment library against the labeled and unlabeled substrates using both detection methods produced three disparate hit sets and hit rates. Follow-up of selected compounds demonstrated the existence of different hit classes among the assays. Interestingly, FS and MS produced different hit sets when used as complementary detection methods on the same samples. While some MS hits (including the most potent) were obscured by autofluorescence in the FS assay, this phenomenon alone did not fully account for the discrepancy between techniques. MS also generated different hit sets for the labeled and the unlabeled peptide, finding both hits that were active against the labeled peptide but not the unlabeled, and *vice versa*. The existence of these two populations of compounds underscores the importance of substrate selection when setting up a new screen.

Pairing the RapidFire high-throughput system with MS solves the time bottleneck associated with MS detection, allowing an analysis rate of approximately 10 seconds per sample, and thus approaching the speeds of fluorescent plate readers. Label-free screening by high-throughput MS has proven to be a valid method for conducting activity-based screens of fragment libraries that enables the study of more native molecules and is less susceptible to confounding factors, such as autofluorescence.

Reference

1. http://www.emdmillipore.com/life-science-research/beta-secretase-inhibitor-iv/EMD_BIO-565788/p_moKb.s1OGx8AAAEjBopJNLpP, accessed 08/21/**2012**.

www.agilent.com/lifesciences/rapidfire

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