A Miniaturized 1536-Well Format γ -Secretase Assay

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ABSTRACT

γ -Secretase is an aspartyl protease that cleaves multiple substrates including the amyloid precursor protein (APP) and the Notch proteins. Abnormal proteolysis of APP is involved in the pathogenesis of Alzheimer's disease (AD) and overactive Notch signaling plays an oncogenic role in a variety of cancers. γ -Secretase has emerged as a promising target for drug development in the treatment of AD and cancer. Assays with increased capacity for high-throughput screening would allow for quicker screening of chemical libraries and facilitate inhibitor development. We have developed a homogeneous time-resolved fluorescence (HTRF)-based assay that makes use of a novel biotinylated recombinant APP substrate and solubilized membrane preparation as the source of the γ -secretase enzyme. The assay was miniaturized to a 1536-well format and validated in a pilot screen against a library of ~3,000 compounds. The overall assay performance was robust due to a calculated Z' factor of 0.74 and its demonstrated ability to identify known γ -secretase inhibitors such as pepstatin A. This validated assay can readily be used for primary screening against large chemical libraries searching for novel inhibitors of γ -secretase activity that may represent potential therapeutics for AD and a variety of neoplasms.

INTRODUCTION

β -Amyloid peptides (A β) are derived from the processing of the amyloid precursor protein (APP) through sequential proteolysis by β -secretase and γ -secretase. According to the "amyloid cascade hypothesis," the β -amyloid peptides contribute to the neurodegeneration associated with Alzheimer's disease (AD).¹ γ -Secretase is a multiprotein complex composed of at least four proteins: PEN2,² APH-1,^{2,3} nicastrin,⁴ and presenilin.^{5,6} This enigmatic protease provides an appealing drug target due to its role in the production of the various β -amyloid species. In addition to initiating proteolysis of APP, γ -secretase also regulates the cleavage of Notch,⁷ ErbB-4,⁸ CD44,⁹ and the Notch ligands Delta-1 and Jagged-2.^{10,11} Due to the role of γ -secretase in AD, a great deal of effort has been directed toward γ -secretase inhibitor (GSI) development for therapeutic benefit.¹² Numerous GSIs have been identified and developed with significant potencies¹²; however, they have been plagued by inhibition of γ -secretase-mediated Notch signaling. It was determined that inhibition of Notch signaling in the small intestine epithelium by GSI treatment resulted in disruption of the progenitor crypt population.¹³ By altering the intestinal epithelium, GSI treatment often results in gut toxicity in patients.¹⁴ Recently, a Notch-1 sparing GSI was reported for the treatment of AD that exhibited selectivity for inhibition of A β 40 and A β 42 over Notch.¹⁵ This highlights the possibility of developing GSIs that possess inhibitory selectivity and can distinguish between the various cleavage activities of γ -secretase. The likelihood of developing selective inhibitors will depend on the discovery of diversified scaffolds, which would greatly enhance the possibility of successful therapeutic application of GSIs.

Numerous biochemical assays have been developed for the characterization of γ -secretase.^{6,16,17} In addition, various cell-based assays have been established that utilize reporter systems^{18,19} or

ABBREVIATIONS: A β , β -amyloid; AD, Alzheimer disease; APP, amyloid precursor protein; CHAPSO, 3-[[3-(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate]; CTF, C-terminal fragment; ECL, electrochemiluminescence; GSI, γ -secretase inhibitor; HTRF, homogeneous time-resolved fluorescence; MBP, maltose-binding protein; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

detect secreted A β species from cells.^{20–22} Most of these assays are not suited for high-throughput screening although few of the cell-based assays have been adapted into a 384-well format for screening chemical libraries.^{19,22} However, miniaturization of a biochemical γ -secretase assay using a recombinant substrate has not been reported for library screening due to instrumentation limitations and/or reagent sensitivity. The development of such a miniaturized 1536-well formatted assay will allow for the rapid screening of continually expanding chemical libraries for GSIs, as well as provide a more cost-effective option for analysis of these large libraries.

Considering the limitations associated with the methods described earlier, we have generated a novel recombinant APP substrate and developed a homogeneous time-resolved fluorescence (HTRF)-based assay for γ -secretase activity. We have demonstrated that our biotinylated substrate exhibits significantly enhanced activity over previously reported substrates.^{6,23} Furthermore, the introduction of this substrate has allowed for the miniaturization of the γ -secretase assay to a 1536-well format with a Z' factor of 0.74 using HTRF detection in a total volume of 10 μ L. This optimized assay has considerably reduced the amount of reagents required for a typical screening campaign of a large compound library, making it exceedingly cost effective and requiring minimal time to perform a screen.

MATERIALS AND METHODS

Overexpression and Purification of Sb4 Substrate

A DNA fragment encoding amino acids 620–695 of the 695-aa isoform of the APP substrate and a maltose-binding protein (MBP) tag sequence at the N-terminus was cloned into the prokaryotic expression vector pIAD16-Avi vector. The recombinant protein, Sb4, was overproduced in *Escherichia coli* (strain BL21(DE3)) simultaneously with the pACYC184 plasmid containing an IPTG-inducible *BirA* gene to express biotin ligase. Expression of Sb4 protein, as well as biotin ligase, was induced using 0.1 mM IPTG for 5 h at 20°C. This was done in the presence of 50 μ M biotin (Fisher Scientific, Pittsburgh, PA) to allow biotinylation of Sb4. *E. coli* cells were pelleted at 8,000g for 30 min, mechanically homogenized by French Press (Spectronic Instruments, Rochester, NY), and supernatants collected following a 30-min spin at 17,000g. Sb4 was subsequently purified by applying the supernatant to an amylose resin using Amersham Biosciences AKTApriime chromatographic system (Piscataway, NJ). Fractions containing Sb4 were collected and thrombin-cleaved to remove the MBP tag.

Membrane Preparation and Solubilization

HeLa membrane was prepared and solubilized as described previously.⁶ In brief, HeLa cell pellet was resuspended in 1 $\times$ 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (50 mM MES,

pH 6.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂) and treated with “complete” protease inhibitor (Boehringer, Mannheim). The resuspended pellet was mechanically homogenized by passing the cells through the French Press (Spectronic Instruments), and subsequently spun down at 800g to remove cell debris and nuclei. Next, the supernatants were spun at 100,000g in order to pellet the membranes. The pelleted membranes were resuspended in 1 $\times$ MES buffer to ~12 mg/mL. This entire procedure was performed at 4°C. HeLa membranes were solubilized using 1% CHAPSO detergent in 1 $\times$ MES buffer with protease inhibitors. After incubating for 1 h at 4°C, the membrane was spun down at 100,000g and the supernatants were collected and stored at –70°C. The collected fraction is defined as solubilized γ -secretase.

γ -Secretase Assay Evaluation Using Electrochemiluminescence Detection

The *in vitro* γ -secretase assay used to evaluate the Sb4 substrate was performed similarly as previously reported.⁶ In brief, Sb4 recombinant substrate was incubated with 40 ng/ μ L solubilized γ -secretase in the presence of 0.25% CHAPSO detergent. This was incubated for 2.5 h at 37°C. Reaction supernatant was then incubated with ruthenylated G2-10* antibody in phosphate-buffered saline (PBS) + 0.5% Tween for 2 h at room temperature. Streptavidin beads were added and incubated for an additional 30 min. Finally, electrochemiluminescence (ECL) was quantified on a reader (Bioveris, Gaithersburg, MD).

Dose-Response Studies

In each assay, the signal inhibition induced by the compounds was expressed as a percentage compared to high and low controls located on the same plate, as defined as percentage inhibition = (high control average – read value)/(high control average – low control average) $\times$ 100. The dose response was assessed in duplicate using 12-point doubling dilutions with 100 μ M compound concentration as the upper limit. The dose–response curve for each data set was individually fitted, and the two IC₅₀ values obtained were subsequently averaged.

Statistical Analysis

The Z' factor was used to assess assay performance. The Z' factor constitutes a dimensionless parameter that ranges from 1 (infinite separation) to <0. It is defined as: $1 - Z' = (3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c+} - \mu_{c-}|$, where σ_{c+} , σ_{c-} , μ_{c+} , and μ_{c-} are the standard deviations (σ) and averages (μ) of the high (c_+) and low (c_-) controls.²⁴

Chemical Libraries

The library used for the pilot screen combines ~3,000 chemicals obtained commercially from Prestwick and MicroSource. Biotin was

included in the MicroSource library. The MicroSource library contains 2,000 biologically active and structurally diverse compounds from known drugs, experimental bioactives, and pure natural products. The library includes a reference collection of 160 synthetic and natural toxic substances (inhibitors of DNA/RNA synthesis, protein synthesis, cellular respiration, and membrane integrity), a collection of 80 compounds representing classical and experimental, pesticides, herbicides, and endocrine disruptors, as well as a unique collection of 720 natural products and their derivatives.

Additionally, the collection includes simple and complex oxygen containing heterocycles, alkaloids, sesquiterpenes, diterpenes, pentacyclic triterpenes, sterols, and many other diverse representatives. The Prestwick Chemical library is a unique collection of 880 high-purity chemical compounds (all off-patent) and carefully selected for: structural diversity, and a broad spectrum covering several therapeutic areas (from neuropsychiatry to cardiology, immunology, anti-inflammatory, analgesia, and more), known safety, and bioavailability in humans. Over 85% of its compounds are marketed drugs. Biotin was also included in the Prestwick library.

Automation System and Screening Data Management

The 384-well validation and 1536-well format screening assays were performed on a fully automated linear track robotic platform (CRS F3 Robot System, Thermo Electron, Canada) using several integrated peripherals for plate handling, liquid dispensing, and fluorescence detection. Two different liquid dispensing devices were used in this study. Compounds were plated at a volume of 1 μ L for the HTRF assay using a TPS-384 Total Pipetting Solution (Apricot Designs, Covina, CA). Dispensing of γ -secretase and HTRF mix was performed using the Flexdrop IV (Perkin Elmer, Waltham, MA). The HTRF mix contained both a europium-labeled anti-mouse antibody along with streptavidin conjugated to a trimeric form of allophycocyanin referred to as XL665 (Cisbio Biosystems, Bedford, MA).

Detection of HTRF was measured with the Perkin Elmer VICTOR3 VTM Multi label counter. Screening data files from the VICTOR3 VTM were loaded into the HTS Core Screening Data Management System, a custom-built suite of modules for compound registration, plating, data management, and powered by ChemAxon Cheminformatic tools (ChemAxon, Hungary). Data was analyzed for compounds exhibiting 49.5% inhibition or greater, and the summary of the identified positives was exported as SD files for further analysis and reporting.

RESULTS

Evaluation of Novel γ -Secretase Substrate Using Electrochemiluminescence Detection

Previously published γ -secretase assays have used a truncated APP protein, C100Flag, which requires biotinylated 4G8 antibody

directed to the substrate, in addition to G2-10 antibody that binds cleaved substrate.^{6,25} G2-10 specifically recognizes the A β 40 processed site of the cleaved substrate,²⁵ but does not bind to uncleaved substrate. Therefore, we attempted to eliminate the use of biotinylated antibody for detection of γ -secretase by incorporating an Avitag directly into the substrate. Avitag, a conserved peptide sequence, is recognized by biotin ligase that specifically catalyzes an attachment of biotin to the lysine residue within the tag during target protein expression in *E. coli*.²⁶ We constructed a double-tagged (Avitag and MBP) recombinant protein that facilitated protein purification and cleaved product detection. This protein also contains a thrombin cleavage site between MBP and Avitag. After purification, the MBP is removed by thrombin cleavage and biotinylated C-terminal fragment (CTF), referred to as Sb4, is isolated as a γ -secretase substrate. The identity and biotinylation of Sb4 has been confirmed by LC-MS analysis and we determined that ~90% of Sb4 protein is biotinylated (data not shown). Sb4 or C100Flag at 1 μ M was incubated with γ -secretase in the absence or presence of 100 nM of the potent GSI Compound E (Table 1),^{27,28} and these samples were referred to as the high and low controls, respectively. The γ -secretase-cleaved A β product derived from C100Flag substrate was detected by a pair of antibodies (biotinylated 4G8 and ruthenylated G2-10*), while Sb4-cleaved product was detected by ruthenylated G2-10* alone (Table 1) and quantified using ECL technology. The ratio of signal to background for Sb4 and C100Flag were 93:1 and 7:1, respectively (Table 1). Clearly, this newly engineered substrate exhibits significantly increased assay sensitivity for measuring *in vitro* γ -secretase activity while making use of only one antibody for product detection. However, the ECL γ -secretase assay is heterogeneous in nature and is not amenable to further miniaturization; therefore, it is not suitable for screening large libraries. In order to advance the γ -secretase assay into higher plate density formats, we incorporated an HTRF detection method.

Development of an HTRF-Based Assay for γ -Secretase Activity in 384-Well Format

We set out to transfer the assay conditions obtained with the ECL method to an HTRF detection-based format (Fig. 1). The HTRF detection mixture consisted of mouse G2-10 antibody for cleaved product recognition, an europium-labeled anti-mouse antibody, along with streptavidin conjugated to a trimeric form of allophycocyanin referred to as XL665. Cleavage of Sb4 substrate allows G2-10 mouse antibody to bind to the A β 40-processed site on cleaved substrate. The streptavidin-XL665 entity interacts directly with the biotin moiety on the Sb4-derived product. Finally, the anti-mouse europium antibody targets to the G2-10 antibody, bringing all of these entities

Table 1. Comparison of the Reactivity of Sb4 and C100Flag in a γ -Secretase Assay Utilizing Electrochemiluminescence (ECL) Detection

Substrate	Activity (ECL units/min/ μ g)		Signal:noise
	High	Low	
Sb4 ^a	345 $\pm$ 27	3.73 $\pm$ 0.06	92.5
C100Flag ^b	22.5 $\pm$ 2.6	3.37 $\pm$ 0.06	6.7

The C100Flag substrate is not biotinylated, and therefore requires an additional biotinylated antibody in the assay. High activity was defined in the presence of 1% dimethyl sulfoxide (DMSO) (v/v), whereas low activity is the remaining γ -secretase activity in the presence of 100 nM Compound E γ -secretase inhibitor (delivered in 1% DMSO (v/v)). Each assay point was performed in quadruplicate and standard deviation is depicted.

^aSingle antibody detection assay (Ru-G2-10).

^bDual antibody detection assay (Biotin-4G8 and Ru-G2-10).

into close proximity. The europium and XL665 reagents are a compatible pair of donor-acceptor fluorophores, and excitation of europium at 337 nm results in release of light at 620 nm by europium, which then diffuses to stimulate XL665. Ultimately, streptavidin-XL665 converts 620 nm to release quantifiable light at 665 nm (Fig. 1). HTRF conditions were initially optimized in a 384-well format using a final volume of 20 μ L. Again, the high control for this assay was defined as the observed γ -secretase activity in the presence of 1% dimethyl sulfoxide (DMSO) (v/v) and the low control as the activity observed when including 100

nM final concentration of Compound E (v/v). First, we examined the tolerable DMSO concentration that would not interfere with γ -secretase activity. Even with 3% DMSO (v/v) present in the assay mixture, there was virtually no effect on the γ -secretase activity (Fig. 2A), which was critical as delivery of library screening compounds required a final concentration of 2% DMSO (v/v) in the γ -secretase reaction. Next, we titrated the HTRF mixture components in an effort to minimize screening costs. A high signal was witnessed at 1 nM G2-10; however, we chose a final concentration of 0.3 nM that exhibited the desired 4:1 signal to noise ratio (Fig. 2B). Additionally, we examined europium-conjugated anti-mouse antibody and determined that 1 nM in the HTRF mixture was sufficient for our assay conditions even though the signal continued to increase with higher concentrations of the anti-mouse antibody (Fig. 2C). Finally, using this optimized assay (2% DMSO (v/v), 0.3 nM G2-10 and 1 nM europium-conjugated anti-mouse antibody), we examined the IC₅₀ values of the two GSIs L-685,458 and Compound E and determined as 1.4 and 1.1 nM, respectively, in our HTRF assay, which is consistent with data using the ECL assay (Fig. 2D).

Fig. 1. Development of a homogeneous time-resolved fluorescence (HTRF) γ -secretase assay using a biotinylated recombinant substrate Sb4. Schematic representation of HTRF γ -secretase assay. Cleavage of Sb4 substrate by γ -secretase at the A β 40 site is detected using G2-10 antibody that binds only cleaved substrate. HTRF detection method is incorporated to quantify enzyme activity. Anti-mouse IgG-linked europium fluorophore binds to the G2-10 antibody, and streptavidin-conjugated XL665 fluorophore binds at the biotinylated portion of Sb4. Following cleavage of Sb4, all reaction entities come into close proximity allowing FRET to occur. Europium is stimulated with light at 337 nm, causing the release of a signal at 620 nm. The 620 nm light stimulates XL665 fluorophore to release light at 665 nm and this is quantified on a plate reader. Data values are calculated as the fraction of 665 nm signal normalized to background 620 nm.

γ -Secretase Assay Miniaturization to a 1536-Well Format and Pilot Screening

We have further miniaturized the HTRF assay from the established 384-well format to a 1536-well platform enabling more efficient screening of large chemical libraries. We showed that miniaturization did not lead to any apparent signal distortion when comparing biochemical activity from the 384- and 1536-well formats (Fig. 2E) and determined that both

Fig. 2. Optimization of homogeneous time-resolved fluorescence (HTRF) γ -secretase assay for utilization in a large-scale high-throughput screening. **(A)** Titration of tolerable levels of dimethyl sulfoxide (DMSO) in the γ -secretase reaction portion of the HTRF assay, ranging from 0% to 20% DMSO or no γ -secretase enzyme (indicated as No GS). Due to the allotment of 1 μ L of high-throughput compounds in 10% DMSO for 1536-well screening, the γ -secretase assay needed to withstand a final DMSO concentration of 2% (v/v). **(B)** Optimization of G2-10 antibody concentration for characterization of cleaved substrate. **(C)** Anti-mouse IgG-conjugated fluorophore was titrated. **(D)** Inhibitory potency of L685,458 (●) and Compound E (■) determined by the HTRF γ -secretase assay. Each assay point was performed in triplicate, and SD is plotted. **(E)** Optimization conditions were performed in a 384-well assay format (black bars) with a final reaction volume of 20 μ L. We finalized assay parameters to 0.3 nM G2-10 and 1 nM anti-mouse IgG europium cryptate. Finally, the assay was miniaturized to a 1536-well format (gray bars) in a 10 μ L reaction volume (5 μ L γ -secretase reaction mix + 5 μ L HTRF detection mix). **(A)–(C), (E)** For each assay point, $n = 8$ and SD is plotted.

formats exhibit similar signal to background ratios. Based on these optimization experiments, we set our final assay conditions at 0.3 nM G2-10, 1 nM anti-mouse antibody, 15 nM streptavidin-XL665, and proceeded with the assay at 10 μ L final volume (5 μ L γ -secretase mix + 5 μ L HTRF mix) in the 1536-well format for screening.

Following the successful establishment of assay conditions in a 1536-well format, we progressed to validate this newly miniaturized γ -secretase assay in a pilot screen against a library of ~3,000 compounds. As part of this validation step, we first performed a control run consisting of two 1536-well plates; one as a high control plate representing γ -secretase activity in 1% DMSO (v/v) and the other as a low control plate representing residual activity of fully inhibited γ -secretase using 100 nM Compound E GSI in 1% DMSO (v/v). Figure 3A and B depicts the control run results and reveals excellent separation between high and low control wells resulting in a Z' factor of 0.74 and a signal to noise ratio of 4:1. The calculated coefficients of variation (CV) for both the high and low controls were 4.96% and 6.78%, respectively (Fig. 3B). These data demonstrated that the assay was very stable even when fully automated and possessed minimal well-to-well variability; therefore, we proceeded with the pilot screen of 3,000 validation library compounds. The pilot screen was carried out at a single dose of 10 μ M for each library compound in 1% DMSO (v/v) using the same conditions that will be utilized for a full-scale high-throughput screening campaign (e.g., same robotic platform, readers, reagents, etc.). The pilot assay was performed in duplicate on two separate days to account for any day-to-day variability. This allowed us to obtain field data on assay performance, assay sensitivity for identifying

inhibitors, an estimate of the initial hit rate, an overall assessment of compound interference, and most importantly, an evaluation of assay reproducibility by comparing the two individual data sets from each screening. The initial hit rate of the pilot screen was 1.1% and was consistent with previously screened *in vitro* assay targets at the MSKCC HTS Core Facility. This similar hit rate was likely because these two validation libraries contain several pan-active compounds that have the potential to act as promiscuous active agents in the assay.^{29,30} The Z' values per

Fig. 3. A β 40 homogeneous time-resolved fluorescence (HTRF) assay pilot screen results. (A) Distribution of high and low control wells from the control run using one 1536-well plate representing high control wells and one 1536-well plate representing low control wells. High control wells contain 1% dimethyl sulfoxide (DMSO) (v/v), whereas low control wells contain 100 nM Compound E γ -secretase inhibitor (GSI) in 1% DMSO (v/v). (B) Frequency distribution of the high and low control wells yielding a Z' value of 0.74 and a signal to noise ratio of 4:1. (Continued)

plate were consistent with those obtained during the original control run with Z' values of 0.75 or 0.76 from the four 1536-well duplicate assay plates. The scatter plot of the screen performed on two subsequent days demonstrates excellent reproducibility between the 2 days (Quadrants I and III) with the majority of inactive compounds centered on the zero axis and an estimated assay noise of 25% (Fig. 3C). Figure 4 summarizes a few of the active

compounds obtained from this pilot screen. Among them, biotin was twice identified as an active hit in the pilot screen because it was present in both the Prestwick chemicals and MicroSource libraries (Fig. 4). The substrate used in our assay is biotinylated and the detection step employs streptavidin-conjugated fluorophore; therefore, excess biotin disrupts the streptavidin-XL665 interaction with substrate (Fig. 1). Cisplatin, a platinum-based chemotherapeutic drug used to treat various cancers, results in DNA cross-linking and leads to induction of cell death through apoptosis. It was identified as an active compound in the pilot screen likely due to its ability to quench the europium fluorescence signal, and is an example of a promiscuous active agent in this γ -secretase assay (Fig. 4). Pepstatin A, a biologically relevant and well-characterized inhibitor of aspartic acid proteases,^{6,31} was identified as an active compound by our newly miniaturized assay during the pilot screen (Fig. 4). We subjected pepstatin A to a dose-response study in order to establish its potency against γ -secretase in our assay and determined that it inhibits the enzyme with a calculated IC_{50} of 6.43 μM (Fig. 3D).

DISCUSSION

β -Amyloid peptides are produced by the processing of APP first by β -secretase and subsequently by γ -secretase. Both secretases have attracted a great deal of attention as potential drug targets for inhibitor development. Screening assays to identify inhibitors of β -secretase that employ HTRF technology have previously been reported.^{32,33} The development of selective inhibitors against γ -secretase that abrogate processing

Fig. 3. (Continued) (C) Scatter plot analysis from the A β 40 HTRF pilot screen. A library of ~3,000 compounds was screened on two successive days to evaluate assay reproducibility and performance. Strong positives in Quadrant I represent potential GSIs. Data points located in Quadrant II or IV are compounds that did not reproduce in both runs of the assay. The majority of compounds located around the X- and Y-axis intercept represent compounds that did not exhibit any significant activity against γ -secretase. (D) Dose-response curve of pepstatin A. Data points were performed in duplicate.

Fig. 4. Structures of a few promiscuous compounds identified in the homogeneous time-resolved fluorescence (HTRF)-based γ -secretase pilot screen.

of APP over Notch and other substrates may provide advantages for effective AD therapies. Recently, Notch-1-sparing GSIs were reported that inhibit A β 40 and A β 42, yet possessed significantly reduced potency for abrogation of Notch signaling.¹⁵ These studies indicate that it is possible to preferentially distinguish between the varied cleavage activities of γ -secretase, but also highlight the need for further development of potent and selective GSIs. Our newly developed and miniaturized A β 40 HTRF assay will facilitate the screening and identification of novel inhibitor scaffolds from large chemical libraries.

The presented 1536-well HTRF assay represents a significantly improved platform for screening large libraries of small molecule inhibitors of γ -secretase and provides numerous advantages over existing assays. First, this is the only reported miniaturized γ -secretase assay making use of a recombinant and biotinylated APP substrate. This assay allows for the direct identification of GSIs using a solubilized membrane fraction enriched for the enzyme and eliminating any residual effects or artifacts from *in situ* cellular lysis, thus avoiding complicated cellular

environmental effects. Second, the use of a biotinylated substrate eliminates the need to employ a dual antibody detection strategy that requires both biotinylated antibody against β -amyloid as well as antibody targeted to the substrate cleavage site. The G2-10 antibody targeted to the A β 40 cleavage site has been widely used for monitoring the production of A β products and for quantifying β - and γ -secretase activities.^{20–22} Third, the simplicity of our engineered substrate and its higher sensitivity has allowed the assay to be miniaturized to a 1536-well format. This provides substantial savings on the cost of detection reagents as well as screening-associated expenses. Lastly, this newly miniaturized assay has proven to be highly robust and reproducible as indicated by the Z' value of 0.74. It is also sensitive enough to identify modulators of γ -secretase activity like pepstatin A, one such compound with micromolar activity. Taken together, the development of our 1536-well format assay will allow for rapid screening of large chemical libraries for GSIs that suppress A β 40 production. Its successful validation in a pilot screen of ~3,000 compounds has demonstrated its sensitivity for identification of active and

structurally diverse chemicals such as pepstatin A with an IC₅₀ value of 6.43 μ M.

Coupling this method with other low or medium throughput assays for A β 42 production or Notch cleavage by γ -secretase may be effective for identifying selective GSIs. Using this type of coupled screening approach will greatly increase the opportunity to identify selective inhibitors and facilitate the development of effective γ -secretase-targeted therapeutics that eliminates severe clinical toxicities that have previously been witnessed. In conclusion, this 1536-well *in vitro* assay format represents a significant advancement in the ability to rapidly screen for novel small molecule inhibitors of γ -secretase and will facilitate the drug discovery process of novel and more selective therapies for neurodegenerative diseases and cancer.

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