Published in final edited form as:

Comb Chem High Throughput Screen. 2013 July 1; 16(6): 415–424.

A Novel High-Throughput 1536-well Notch1 γ -Secretase AlphaLISA Assay

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Abstract

The Notch pathway plays a crucial role in cell fate decisions through controlling various cellular processes. Overactive Notch signal contributes to cancer development from leukemias to solid tumors. γ -Secretase is an intramembrane protease responsible for the final proteolytic step of Notch that releases the membrane-tethered Notch fragment for signaling. Therefore, γ -secretase is an attractive drug target in treating Notch-mediated cancers. However, the absence of high-throughput γ -secretase assay using Notch substrate has limited the identification and development of γ -secretase inhibitors that specifically target the Notch signaling pathway. Here, we report on the development of a 1536-well γ -secretase assay using a biotinylated recombinant Notch1 substrate. We effectively assimilated and miniaturized this newly developed Notch1 substrate with the AlphaLISA detection technology and demonstrated its robustness with a calculated Z' score of 0.66. We further validated this optimized assay by performing a pilot screening against a chemical library consisting of ~5,600 chemicals and identified known γ -secretase inhibitors e.g. DAPT, and Calpeptin; as well as a novel γ -secretase inhibitor referred to as KD-I-085. This assay is the first reported 1536-well AlphaLISA format and represents a novel high-throughput Notch1- γ -secretase assay, which provides an unprecedented opportunity to discover Notch-selective γ -secretase inhibitors that can be potentially used for the treatment of cancer and other human disorders.

Keywords

Alzheimer disease; AlphaLISA; cancer; γ -secretase; γ -secretase modulators; Notch signaling

INTRODUCTION

γ -Secretase is a multi-subunit intramembrane aspartyl protease consisting of at least four known major components including Presenilin (PS), Nicastrin, Pen2 and Aph.[1] γ -Secretase cleaves an array of type I membrane proteins including the amyloid precursor

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protein (APP) and Notch receptors. APP is cleaved by β -secretase and γ -secretase to produce β -amyloid peptides (A β) that aggregate to form plaques in Alzheimer's disease (AD) patient's brain, a hallmark of AD.[2] Notch signaling, which is evolutionarily conserved from *C. elegans* to human, plays a significant role in cell fate determination by controlling cell differentiation, proliferation, and apoptosis.[3] The Notch signaling cascade is initiated when receptor-bearing cells interact with Notch ligands expressed on adjacent cells. The binding of ligand to Notch triggers two proteolytic events, first, by ADAM17 or ADAM10 and, second, by γ -secretase. Following γ -secretase cleavage, the Notch intracellular domain (NICD) is released from the membrane tether and translocates to the nucleus, where it activates transcription of target genes (Figure 1A).[4] There are four mammalian Notch receptors (Notch1-4) and five Notch ligands (Dll-1, -3, -4, Jagged-1, -2). [5] All four receptors have been linked to cancer and other human disorders.[6-9] Therefore, γ -secretase is an appealing drug target for controlling the production of A β and NICD for AD and cancer therapy, respectively. Indeed various γ -secretase inhibitors and modulators are currently in clinical trials for either AD or cancer treatments. However, the wide spectrum of γ -secretase substrates has made developing γ -secretase-based therapies a formidable challenge, exemplified by the recently failed Phase III clinical trial of Semagacestat, a non-selective γ -secretase inhibitor (GSI). Therefore, the selective mechanism of these drugs for inhibiting either APP or Notch cleavage remains to be further elucidated. These studies also demonstrate the importance of developing selective γ -secretase inhibitors that will limit undesired side effects.

One of the main challenges in developing substrate-selective γ -secretase inhibitors is the lack of high-throughput biochemical assay that can be used for rapid drug screening. Extensive effort has been made to develop γ -secretase assays for biochemical and cellular characterization of this extraordinary intramembrane proteolysis and for screening inhibitors.[10-18] Among these assays, a 1536-well formatted *in vitro* γ -secretase assay with a recombinant APP substrate was used to rapidly screened chemical libraries to discover γ -secretase inhibitors (GSIs).[18],[19] Although similar biochemical assays using Notch as substrates have been developed, these assays rely on either western blot analysis or mass spectrometry for the detection of Notch cleavage product. Such assays are extremely limited in its capability for high-throughput drug screening and prevented the identification of novel Notch-specific GSIs through this method. In addition, the lack of robust and quantitative *in vitro* γ -secretase assay using Notch substrates has hampered the quick determination of the specificity of γ -secretase inhibitors. Recently, we have devised an *in vitro* γ -secretase assay and applied it to investigate the effects of Familial Alzheimer's disease-associated PS mutations on Notch cleavage [20] and to characterize Notch-sparing inhibitors.[21] In this assay, a novel biotinylated recombinant Notch1 substrate (N1-Sb1), a γ -secretase cleavage-dependent antibody SM320 and AlphaLISA technology [22] were used. However, whether this assay can be miniaturized into a 1536-well AlphaLISA platform for inhibitors screening remains to be explored.

In this study, we optimized and miniaturized the γ -secretase AlphaLISA assay [20] for Notch1 cleavage into a 1536-well format that has a total assay volume of 10 μ l. This high throughput assay has an optimum Z' factor of 0.66, which we then proceeded with screening a small library consisting of approximately 5,600 compounds. We further validated this assay by successfully identifying known γ -secretase inhibitors such as calpeptin and DAPT. This miniaturized Notch1 γ -secretase assay is highly reproducible, convenient, and cost-effective, which can be used to identify novel γ -secretase inhibitors that target the Notch proteolysis and/or characterize the specificity of known inhibitors.

MATERIALS AND METHODS

Purification of N1-Sb1 substrate and polyclonal SM320 antibodies

The N1-Sb1 substrate was expressed and purified as described.[20] Briefly, the pIAD16-N1-Sb1-Avi, was co-transformed into the BL21 (DE3) *E. coli* strain with the pACYC 184 plasmid, which expresses biotin ligase (*BirA*). The recombinant substrate, N1-Sb1, was induced with 0.1mM IPTG in the presence of 50 μ M biotin for 5 hrs at 20°C. *E. coli* cell pellet was collected by centrifugation at 8000 $\times g$ for 30 mins and the resulting pellet was lysed with a French Press (Spectronic Instruments, Rochester, NY). The lysate was centrifuged for 30 mins at 17,000 $\times g$ and the resulting supernatant was purified with amylose resin. The N1-Sb1 was generated by thrombin treatment to remove the MBP tag. Rabbit SM320 antibodies that specifically recognize the γ -secretase-cleaved product, but not the substrate, was prepared and characterized as described.[20]

Membrane solubilization and γ -secretase AlphaLISA assay

HeLa-S3 cell was purchased from National Cell Culture Center and solubilized membrane was prepared as described.[10,18] Briefly, the pellet was resuspended with 1X MES buffer (50mM MES, pH6.0, 150mM KCl, 5mM CaCl₂, 5mM MgCl₂) containing 'complete' protease inhibitors (PI) (Boehringer Mannheim, Gaithersburg, MA) and lysed with a French Press (Spectronic Instruments, Rochester, NY). Cell debris and nuclei were removed with centrifugation at 800 $\times g$ for 15 mins and the ensuing supernatant was spun at 100,000 $\times g$ for 1 hr. The resulting pellet containing the cell membrane was solubilized in 1X MES buffer with 1% CHAPSO detergent and PI. After 1 hr of incubation, the membrane was centrifuged at 100,000 $\times g$ and the ensuing supernatant contains the solubilized γ -secretase.

The AlphaLISA assay with N1-Sb1 was carried out as previously described.[18,20] After solubilized γ -secretase (40 μ g/ml) was incubated with the N1-Sb1 substrate (0.4 μ M) for 2 hrs at 37°C, the AlphaLISA detection mixture that includes SM320 antibody, Protein A-conjugated acceptor beads, streptavidin-conjugated donor beads and AlphaLISA buffer (25mM HEPES, pH 7.5, 75mM NaCl, 0.05% BSA, 0.05% Tween-20) was added and incubated for 3 hrs. The AlphaLISA signal was detected using Envision plate reader (Perkin Elmer, Waltham, MA)

Chemical libraries

The library used for the screen combines 5,632 compounds obtained from MicroSource, Prestwick, Tocris, and other commercial sources as previously described.[23-28] Briefly, the MicroSource Library contains 2,000 biologically active and structurally diverse compounds. The Prestwick Chemical Library is a unique collection of 1,119 bioactive compounds. The Tocris collection contains 1,270 compounds. The remaining compounds were provided through internal suppliers.

Chemical screen of γ -secretase using the miniaturized and optimized AlphaLISA assay

A library collection of 5,632 compounds containing known drugs and bioactives was screened at a final concentration of 10 μ M test compound in 1% DMSO (v/v) and the screen was performed in duplicate. 1 μ l of 100 μ M compound solution in 10% DMSO (v/v) was transferred from an intermediate 384-well polypropylene microtiter plate (Thermo Scientific, Waltham, MA) to a 1536-well microtiter assay plate (Thermo Scientific). For internal reference, each assay plate contained at a final concentration 1% DMSO (v/v) vehicle only as high control in columns 25-28 in row A-P and 250 nM of Compound E in 1% DMSO (v/v) as low control in columns 25-28 in row Q-AF. To start the assay, 4 μ L of γ -secretase mix was dispensed into the assay plate and incubated for 2 hrs at 37°C to complete the enzymatic reaction. Next, 5 μ L of AlphaLISA detection mix was dispensed into

the assay plate and incubated for 3 hrs at room temperature in the dark after which resulting signal was quantified on the Envision plate reader (Excitation wavelength: 680nm, emission wavelength: 615nm, excitation time: 80ms, total measurement time: 240ms). The optimized assay steps are summarized in Table 1.

Liquid dispensing, automation system, and data management

The screen was performed on a fully automated linear track robotic platform (CRS F3 Robot System, Thermo Scientific) using several integrated peripherals for plate handling, liquid dispensing, and detection systems. Compounds were plated using a custom-designed 384 head on a PP-384-M Personal Pipettor (Apricot Designs, Monrovia, CA). Dispensing of γ -secretase enzymatic reaction and AlphaLISA detection mixture was performed using the FlexDrop IV (Perkin Elmer). Assay plates were incubated in the Steri-Cult automated incubator (Thermo Scientific) at 37°C. Screening data files were obtained from the Envision plate reader. The signal inhibition induced by the compounds was expressed as a percentage compared to high and low controls, which is defined as % Inhibition = (read value – low control average) / (high control average – low control average) $\times$ 100. The screening data files obtained were subsequently loaded into the HTS Core Screening Data Management System, a custom built suite of modules for compound registration, plating, and data management, powered by ChemAxon Cheminformatic tools (ChemAxon, Hungary). The summary of the identified positives was exported as chemical structure data files (SDF) for further analysis and reporting.

Statistical analysis

The Z' factor was calculated from formula: $1 - Z' = (3\sigma_{c+} + 3\sigma_{c-}) / |\mu_{c+} - \mu_{c-}|$ 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.[29] The high signal was determined using control wells containing 1% DMSO (v/v) vehicle only and the low signal using 250nM of Compound E in 1% DMSO (v/v). The Z' factor constitutes a dimensionless parameter that ranges from 1 (infinite separation) to < 0 .

Dose response studies

Calpeptin (Santa Cruz Biotechnology, Santa Cruz, CA) and KD-I-085 (MSKCC HTS) were serially diluted in DMSO and the compounds were assayed in a 384-well γ -secretase assay format as previously described.[18,20] DMSO and Compound E were used as high and low control, respectively. The % Inhibition = (read value – DMSO value) / (DMSO value – Compound E value) $\times$ 100.

RESULTS

Development of 1536-well format γ -secretase AlphaLISA assay

We have developed an *in vitro* γ -secretase assay using the biotinylated recombinant Notch1 substrate (N1-Sb1) and AlphaLISA technology to better characterize γ -secretase.[20] This substrate, which contains the γ -secretase cleavage site (S3 site) (Figure 1C), is initially expressed with an MBP-tag and an Avi-tag. Exogenous biotin is conjugated to N1-Sb1 through the activity of the biotin ligase (*Bir A*), which is concomitantly expressed during protein induction. The MBP-tag is subsequently removed to generate full length N1-Sb1 (Figure 1B). γ -Secretase cleaves N1-Sb1 to produce cN1-Sb1 that has an exposed N-terminal (Figure 1C). SM320 antibody, which specifically recognizes the N-terminal of γ -secretase-cleaved Notch, binds to the N-terminal of cN1-Sb1. A pair of AlphaLISA detection beads is then added to detect cN1-Sb1. Protein A-conjugated acceptor beads bind SM320; while streptavidin-conjugated donor beads bind to the biotin on the C-terminal of cN1-Sb1. These interactions bring the donor and acceptor beads into close proximity. The

excitation of the donor beads leads to the generation of oxygen singlets, which are taken up by the acceptor beads and a chemiluminescence cascade is generated within the acceptor beads to produce light that is detected as AlphaLISA readout (Figure 1C).

We initially examined if solubilized γ -secretase can be used for the activity assay. We found that the CHAPSO-solubilized γ -secretase can effectively cleave N1-Sb1 and display the same characteristics as total membranes (data not shown). To further miniaturize this 1536-well assay for chemical library screen, we focused on three parameters: SM320 antibodies, Protein A-conjugated acceptor beads and streptavidin-conjugated donor beads. First, we compared two concentrations of SM320 antibody – 0.05 μ g/ml and 0.1 μ g/ml, while setting the Protein A-conjugated acceptor beads and streptavidin-conjugated donor beads at 1.25 μ g/ml and 5 μ g/ml, respectively. Although 0.1 μ g/ml of SM320 gave higher AlphaLISA signal, 0.05 μ g/ml of SM320 also showed sufficient readout for further optimization (Figure 2A). Secondly, we titrated the concentration of Protein A-conjugated acceptors beads while fixing the SM320 and streptavidin beads at 0.05 μ g/ml and 5 μ g/ml, respectively. Although higher amount of Protein-A conjugated acceptor beads yielded higher signal (Figure 2B), 0.5 μ g/ml also generated good signal to noise ratio of approximately 11 fold. Finally, we titrated the concentration of streptavidin-conjugated donor beads and we observed that higher streptavidin-conjugated donor beads did not significantly affect the overall assay signal (Figure 2C). These studies showed that a range of concentration of these three reagents with different combinations could generate suitable signal and background ratio for library screening. However, to reduce the cost of reagents, 0.05 μ g/ml SM320, 0.5 μ g/ml Protein A-conjugated acceptor beads, and 2.5 μ g/ml streptavidin-conjugated donor beads were used for the HTS. We tested the reagents at the determined concentrations using 1% DMSO vehicle (v/v) and a previously described γ -secretase inhibitor Compound E [30] and obtained a good screening window for the γ -secretase AlphaLISA assay (Figure 2D).

Control run and screen for inhibitors of γ -secretase for Notch1 cleavage

We assessed the performance and signal window of the γ -secretase AlphaLISA assay in a control run consisting of two 1536-well microtiter plates in the presence of 1% of DMSO (v/v) vehicle only as high control or 250nM of Compound E in 1% DMSO (v/v) as low control. The assay was performed according to the workflow depicted in Table 1. The signal to noise ratio between the high and low control wells in our control run was 7.8 and the Z' factor was 0.66. The box plot showed good signal separation between the high and low controls (Figure 3A). This data demonstrate that the 1536-well microtiter plate format γ -secretase assay is robust and is suitable for HTS.

In order to identify inhibitors of Notch1 using the γ -secretase AlphaLISA assay, we screened a library of 5,632 compounds containing a collection of known drugs, experimental bioactives and natural products. Each compound was screened in duplicate across two separate sets of plates (Set 1 and Set 2) at a final concentration of 10 μ M in 1% DMSO (v/v). High and low controls were included in each plate as internal reference. For Set 1, control wells in plates 1 through 4 resulted in Z' factors of 0.72, 0.58, 0.64 and 0.70, respectively; while Set 2 control wells in plates 1 through 4 resulted in Z' factors 0.70, 0.66, 0.66, and 0.67, respectively. The average Z' factor across the eight plates was 0.67 (Figure 3B), which is consistent with the Z' factor we determined in the control run. Next, we plotted and compared the % inhibition of each compound in set 1 and set 2 from each plate, which show good signal separation between the DMSO signals (blue circles) and Compound E signals (red circles) (Figure 4). More importantly, the % inhibition values between set 1 and set 2 of each plate form a linear correlation. Moreover, this data shows that our γ -secretase AlphaLISA assay in 1536-well microtiter plate is robust and highly reproducible. Finally, we compared the % inhibition values from all the compounds in Set 1 and Set 2 and show a

good linear correlation between both, further suggesting good reproducibility and overall reliability for the screen (Figure 5).

We define positive hits in our study as compounds using a threshold of greater than 50% inhibition and identified 135 compounds as positive for an overall hit rate of 2.4% (red triangles) (Figure 5). Additionally, we identified verteporfin as a stimulator of the assay signal (green triangle) (Figure 5) (Table 2); whereas majority of the compounds are not hits (black dots) (Figure 5). Biotin (Table 3) is one of the hits that we identified and this is an expected artifact since biotin interferes with the binding of streptavidin-conjugated donor beads to the biotin on cN1-Sb1. We also identified cisplatin (Table 3), which is a metal chelator that could potentially interfere with the chemiluminescence cascade within the acceptor beads. More importantly, we identified two known γ -secretase inhibitors – DAPT [31,32], calpeptin [33,34] and a novel γ -secretase inhibitor KD-I-085 (Table 3). We performed dose-titration studies to determine the potencies of calpeptin and KD-I-085 against N1-Sb1 and Sb4, a recombinant APP substrate [35] for γ -secretase cleavage. As expected, calpeptin dose-dependently inhibited N1-Sb1 and Sb4 cleavage with an IC_{50} of $0.24 \pm 0.06 \mu M$ and $0.11 \pm 0.01 \mu M$, respectively (Figure 6A); while KD-I-085 also non-selectively inhibits both N1-Sb1 and Sb4 cleavage with an IC_{50} of $5.32 \pm 0.70 \mu M$ and $4.20 \pm 0.03 \mu M$, respectively (Figure 6B).

DISCUSSION

γ -Secretase is multi-subunit membrane protease that cleaves a wide range of substrates including APP and Notch. APP has important physiological roles in regulating neuron transmission and maintaining the integrity of synapses.[36] On the other hand, Notch signaling plays central role in regulating stem cell differentiation, hematopoietic and neuronal stem cell maintenance. However, aberrant processing of these two substrates has been implicated in either AD or cancer and therefore γ -secretase is an attractive drug target to treat these diseases. A variety of small molecules that target γ -secretase activity have been developed.[37,38] Based on their specificity, γ -secretase inhibitors (GSIs) can be divided into three classes: i) pan γ -secretase inhibitors: This class of GSI, represented by Semagacestat, [39] suppresses γ -secretase activity for all tested substrates and is unlikely desirable for AD therapy development. ii) Notch-sparing γ -secretase inhibitors: these compounds, such as GS-953 [40] and BMS-708163,[41] preferably block γ -secretase activity for A β 40 and A β 42 production over Notch cleavage. iii) γ -Secretase Modulators (GSMs): these compounds selectively inhibit γ -secretase for A β 42 production with a concurrent increase of A β 38 or A β 37, yet have little effect on A β 40 or Notch1 processing.

We have previously reported on a HTS study using APP substrate, Sb4, to screen for novel γ -secretase inhibitors.[18] In that study, we utilized the HTRF technology platform to screen a smaller library of approximately 3,000 compounds. We subsequently screened a larger library, which led to the development of Notch-sparing γ -secretase inhibitor CS-1. [19] Despite the progress made in developing this class of inhibitors, no compounds that selectively targets γ -secretase for Notch1 cleavage has been reported. A major challenge in developing selective γ -secretase inhibitors for Notch1 cleavage inhibition is the lack of high throughput *in vitro* γ -secretase assays that can be used for rapid drug screening. An easy and robust γ -secretase assay using Notch1 substrates has, until now, not been available. Reported γ -secretase/Notch assays rely on western blot analysis or cellular reporter assay, which are impractical for large libraries screening. Recently, a robust *in vitro* γ -secretase assays using a biotinylated Notch substrate, N1-Sb1, in conjunction with the AlphaLISA platform has been reported.[20] We used the AlphaLISA technology in this study for further assay miniaturization and optimization. It has been reported that the Alpha-based technology has high sensitivity and faster plate reading time.[42] In addition, it has also been shown that

5-fold less reagents are needed for Alpha-based assay as compared to the HTRF assays.[42] We successfully miniaturized this assay to a 1536-well format and screened a small library of compounds consisting of 5,632 compounds. To the best of our knowledge, our study is the first report of a 1536-well AlphaLISA assay that is both rapid and highly reproducible, and amenable to high throughput drug screening.

Similar to our previous HTS assay with Sb4,[19] we also uncovered biotin and cisplatin as artifacts. The discovery of verteporfin, an oxygen donor, highlights the potential of detecting compounds that interfere with the AlphaLISA detection reaction instead of the enzymatic reaction directly. In this current HTS, we were able to discover known GSIs such as DAPT and calpeptin. These two compounds were not detected in the early HTS with Sb4 because DAPT and calpeptin were not included in the screening library at that time. In addition to known GSIs, we uncovered a novel GSI KD-I-085, which non-selectively inhibit both Sb4 and N1-Sb1 cleavage. This further validated our assay for screening of larger libraries. More importantly, the combination of this new γ -secretase/Notch1 assay with established γ -secretase/APP assay [18] offer an unprecedented opportunity to identify selective GSIs, such as APP- or Notch-sparing inhibitors. These inhibitors can be used to specifically study the role of Notch and APP independently in biochemical or cellular system. Furthermore, the discovery of selective γ -secretase inhibitors could be valuable for developing effective cancer and Alzheimer's disease therapeutics.

Acknowledgments

The authors wish to thank members of the High Throughput Screening Core Facility for their help during the course of this study. This project is supported by NIH grant AG026660-6A1 (YML), the Geoffrey Beene Cancer Research Center of Memorial Sloan-Kettering Cancer Center (YML) and by Award Number U54CA137788/U54CA132378 from the National Cancer Institute . The HTS Core Facility is partially supported by Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research, the Experimental Therapeutics Center of MSKCC, the William Randolph Hearst Fund in Experimental Therapeutics, the Lillian S Wells Foundation and by an NIH/NCI Cancer Center Support Grant 5 P30 CA008748-44.

Abbreviations

NICD	Notch Intracellular Domain
APP	Amyloid Precursor Protein
MBP	Maltose Binding Protein
GSI	γ -Secretase Inhibitor
PS	Presenilin
IPTG	Isopropyl β -D-1-thiogalactopyranoside
CHAPSO	3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate
MES	2-(N-morpholino)ethanesulfonic acid
DMSO	Dimethyl Sulfoxide
HTS	High-Throughput Screening
PI	“complete” protease inhibitor
BSA	Bovine Serum Albumin
Aβ	β -Amyloid

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A)

B)

Figure 1. Schematic representation of Notch signaling pathway, production of N1-Sb1 and *in vitro* γ -secretase AlphaLISA assay

(A) Notch receptor is activated by the binding of Notch ligands expressed on adjacent cells resulting in the cleavage of Notch by metalloprotease ADAM10 or ADAM17, followed by γ -secretase. γ -Secretase cleaves Notch to produce NICD, which translocates into the nucleus to activate gene transcription. (B) Notch1 fragment is cloned between maltose binding protein gene (MBP) and Avi-tag. The construct is induced with IPTG in the presence of exogenous biotin. MBP-tagged and biotinylated N1-Sb1 is affinity purified and the MBP-tag is removed with thrombin cleavage to generate biotinylated N1-Sb1. (C) N1-Sb1 is cleaved by solubilized γ -secretase at the S3 site to produce cleaved-N1-Sb1 (cN1-Sb1). SM320 binds to the N-terminal of cN1-Sb1. Protein A-acceptor beads interact with SM320 while streptavidin-donor beads bind to the biotin-tag on cN1-Sb1. This multi-entities interaction brings the AlphaLISA donor and acceptor beads into close proximity that allows for the generation and detection of AlphaLISA signal.

Figure 2. Optimization of γ -secretase AlphaLISA assay with N1-Sb1

(A) SM320 antibody was titrated in the presence of 1.25 $\mu\text{g/ml}$ Protein A-conjugated acceptor beads and 5 $\mu\text{g/ml}$ of streptavidin-conjugated donor beads. (B) Protein A-acceptor beads were titrated where the concentration of SM320 and streptavidin-conjugated donor beads were fixed at 0.05 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$, respectively. (C) Streptavidin-conjugated donor beads were titrated in the presence of 0.05 $\mu\text{g/ml}$ of SM320 and 0.5 $\mu\text{g/ml}$ of Protein A-conjugated acceptor beads. (D) 0.05 $\mu\text{g/ml}$ of SM320, 0.5 $\mu\text{g/ml}$ of Protein A-conjugated acceptor beads and 2.5 $\mu\text{g/ml}$ of streptavidin-conjugated donor beads were selected as the final parameter for the 1536-well γ -secretase AlphaLISA assay. DMSO-vehicle or Compound E was used to define the high and low control signals, respectively. The data presented are means $\pm$ SEM of triplicate wells ($n=3$).

Figure 3. Control run with γ -secretase AlphaLISA assay

High control wells were seeded with DMSO and low control wells were seeded with 250nM of Compound E. (A) Box plot comparing high and low control signals demonstrates ~7 fold signal to noise ratio. (B) Library collection of 5,632 compounds were seeded on 4 assay plates and each compound was screened in duplicate (Set 1 and Set 2). High and low controls were included in each plate and the Z' factor of each plate was determined to be around 0.7.

Figure 4. Chemical screen for Notch1 inhibitors with γ -secretase AlphaLISA assay

A library of 5,632 compounds was screened in duplicate assay plates (set 1 and set 2) to determine the reproducibility of the HTS. The % inhibition of each compound was determined and depicted in black circles while signals generated from control wells for either DMSO or Compound E were depicted as blue circles or red circles, respectively.

Figure 5. Scatter plot analysis from γ -secretase AlphaLISA assay

The % inhibitions of each compound that was screened in two sets of plates were compared. Three classes of compounds are showed in this scatter plot – i) non-hits (black circles), ii) positive hits (red inverted triangles), and iii) activator (green triangle).

A)

B)

Figure 6. Dose response studies of Calpeptin and KD-I-085

Calpeptin and compound KD-I-085 were both identified as positive hits in the screen. The potencies of these two compounds were determined in a dose-titration study with the *in vitro* γ -secretase AlphaLISA assay using either Notch substrate (N1-Sb1) or APP substrate (Sb4). The data presented are means $\pm$ SEM of triplicate wells (n=3).

Table 1Workflow of optimization γ -secretase AlphaLISA assay.

Step	Parameter	Value	Description
1	Library compounds	1 μ L	100 μ M in 10% DMSO (v/v)
2	γ -Secretase mix	4 μ L	Enzymatic reaction mix containing 40 μ g/ml solubilized γ -secretase and 0.4 μ M N1-Sb1 substrate
3	Incubation time	2 hrs	37°C
4	AlphaLISA mix	5 μ L	Detection reaction mix containing 0.05 μ g/ml SM320, 0.5 μ g/ml Protein A-conjugated acceptor beads, and 2.5 μ g/ml streptavidin-conjugated donor beads
5	Incubation time	3 hrs	Room temperature
6	Assay readout	680nm/615nm (excitation/emission)	Envision plate reader

Step	Notes
1	Dispensing on the PP-384-M Personal Pipettor using a custom 384 head; 5 mins per 1536-well microtiter plate
2	Dispensing into assay plate with FlexDrop IV; 45 secs per 1536-well microtiter plate
3	Assay plates stored in the Steri-Cult; an automated incubator
4	Dispensing into assay plate with FlexDrop IV; 45 secs per 1536-well microtiter plate
5	Assay plates stored at room temperature in the dark
6	AlphaLISA chemiluminescence signal quantified with Envision plate reader; 12 mins per 1536-well microtiter plate

Table 2

Activator identified using γ -secretase AlphaLISA assay.

Structure	Compound Name	Supplier	ID	AlphaLISA Signal (A.U.) AVG % Inhibition	Therapy / Uses	Reference
Verteporfin	Prestwick Chemicals	1105	-881	Benzoporphyrin derivative used to treat abnormal blood vessels in the eye. In use under the trade name Visudyne.	[40]

Table 3

Inhibitors identified using γ -secretase AlphaLISA assay.

Structure	Compound Name	Supplier	ID	AlphaLISA Signal (A.U.) AVG % Inhibition	Therapy / Uses	Reference
Cisplatin	MicroSource	1502107	114	For the treatment of various malignancies including carcinomas and lymphomas.	[18]
Biotin	MicroSource	1503009	114	Necessary for cell growth, production, and metabolism. Commonly found in a wide range of food sources.	[18]
Calpeptin	Tocris	488	96	Potent inhibitor of Ca^{2+} -dependent protease. Not approved for clinical use in humans.	[25,26]
DAPT	Tocris	2634	94	Potent inhibitor of γ -secretase and blocks Notch signaling. Not approved for clinical use in humans.	[38,39]
Biotin	Prestwick Chemicals	418	82	Necessary for cell growth, production, and metabolism. Commonly found in a wide range of food sources.	[18]
Cisplatin	Tocris	2251	76	For the treatment of various malignancies including carcinomas and lymphomas.	[18]
methyl 2-(2-(benzyloxy)ethyl)-6-formyl-6-methylcyclohex-1-enecarboxylate	Internal Supplier	KD-1-085	71	Function and use unknown.	