

High-Throughput Mass Spectrometry Screening for Inhibitors of Phosphatidylserine Decarboxylase

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Abstract

A high-throughput mass spectrometry assay to measure the catalytic activity of phosphatidylserine decarboxylase (PSID) is described. PSID converts phosphatidylserine (PS) to phosphatidylethanolamine (PE) during lipid synthesis. Traditional methods of measuring PSID activity are low throughput and unsuitable for the high-throughput screening of large compound libraries. The high-throughput mass spectrometry assay measures phosphatidylserine and phosphatidylethanolamine directly using the RapidFire™ platform at a rate of one sample every 7.5 s. The assay is robust, with an average Z' value of 0.79 from a screen of 9,920 compounds. Of 60 compounds selected for confirmation, 54 are active in dose-response studies. The application of high-throughput mass spectrometry permitted a high-quality screen to be performed for an otherwise intractable target.

Materials and Methods

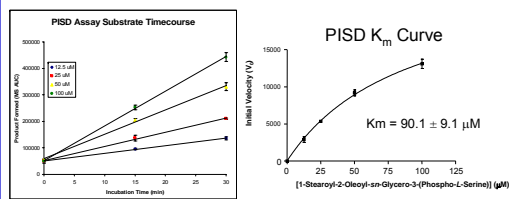
Reagents: 1-Stearoyl-2-Oleoyl-sn-Glycero-3-(Phospho-L-Serine) (PS), and 1-Stearoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (PE) were purchased from Avanti Polar Lipids, Alabaster, AL. PSID corresponds to the full-length human PSID proenzyme (accession number CAG30428) that is self-processed to an active heterodimer. PSID was expressed as a C-terminal FLAG tag (MDYKDDDDK) fusion protein using a pCDNA3.1 plasmid harboring a PCR product encoding residues 1-499 of PSID. The PCR insert was amplified from a human placental library and the coding sequence was confirmed with DNA sequencing. Cell pellets from PSID-transfected HEK 293F cells were dounce homogenized in buffer A (100 mM potassium phosphate, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol). The lysate was centrifuged (500 g) for 10 min and the pellet dounce homogenized in buffer A. The resuspended pellet was centrifuged (500 g) for 10 min. The two supernatants were pooled and centrifuged at 15,000 g for 10 min. The pellet was suspended in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol and stored at -20 °C. The presence of PSID was confirmed by Western blot using an in-house generated antibody. A predominant PSID band was not observed using Coomassie-stained SDS-PAGE, indicating that PSID levels are likely 1% or less. All PSID concentrations are reported as the total protein concentration of the PSID-containing fraction.

Protocol: Primary screening and dose-response data were collected at Bayer Pharmaceuticals Corporation (West Haven, Connecticut, USA) using a subset of the Bayer compound library. Liquid handling was performed with a CyBi-Welt 96 system and a Matrix Technologies Corporation Wellmate. Assays were run at room temperature in 100 mM potassium phosphate, pH 6.8 and 10 mM EDTA. Compound (1 µl) stored in 70% DMSO was transferred into assay buffer (30 µl) in 96-well polypropylene plates (Nunc 249944). The PSID-containing fraction was added to a final concentration of 15 µg/ml (total protein) and the reaction was initiated by adding 50 µl of 40 µM PS in 4% ethanol (final PS concentration, 20 µM). The final reaction volume was 101 µl. The reaction was terminated after 60 min with 20 µl of 417 mM HCl. Plates were sealed, frozen at -80 °C, and shipped on dry ice to Biotrove, Inc. (Woburn, MA) for HTMS analysis. Thawed samples were diluted 100-fold with 100 mM potassium phosphate buffer, pH 6.8, 10 mM EDTA, 0.05% Triton X-100. Sample (5 µl) was delivered directly from 96-well plates to a proprietary clean-up cartridge (BioTrove compound B) to remove non-volatile buffer and membrane components with H₂O in a 2.5 s wash cycle. The substrate and product were co-eluted to the mass spectrometer in 3.5 s using 80% isopropanol. The entire injection cycle was approximately 7.5 s per well. The chromatography system produced baseline-resolved peaks with widths of approximately 3 s.

Substrate Time Course

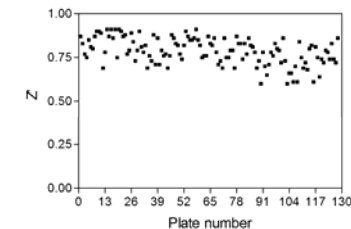
Product formation is linear with time as the substrate concentration is varied ($R^2 > 0.99$). Samples contained 12.5, 25, 50 or 100 µM PS and 25 µg/ml PSID.

The calculated K_m of PS is $90 \pm 9 \mu\text{M}$ ($R^2 > 0.99$).

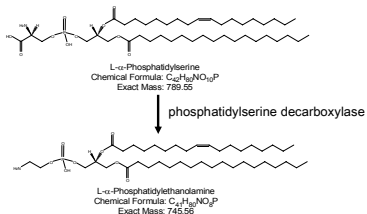


Z' Scores for Individual Assay HTS Plates

The average Z' score of the 124 plates used in the HTS assay was 0.79. Z' values over 0.5 indicate that the assay is robust. Along with 60 test compounds, each assay plate included 8 high control wells (DMSO only) and 8 low control wells (heat-inactivated enzyme) which were used to determine the Z' score.



PSID Reaction Scheme

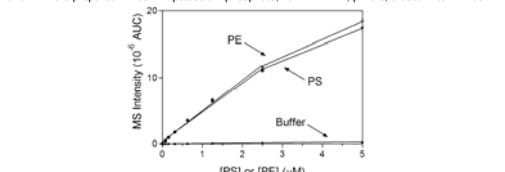


Mass Spectrometry: Direct detection of substrate and product
Established assays: Conversion of ³H-labeled phosphatidylserine to phosphatidylethanolamine and subsequent analysis with thin-layer chromatography, or detection of ¹⁴CO₂ release from ¹⁴C-labeled phosphatidylserine

Limit of Quantitation for PS and PE

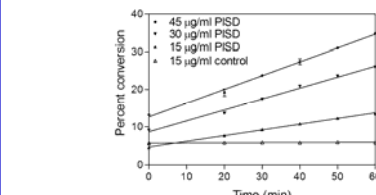
PS and PE were quantified with a Sciex API4000 triple quadrupole mass spectrometer using electrospray ionization in positive ion mode coupled to a RapidFire™ mass spectrometry system. The M+H ions for PS and PE (790.4 and 746.5 amu, respectively) were selected in Q1 while a common fragment at 605.6 amu (corresponding to 1-Stearoyl-2-Oleoyl-glycerol) was selected in Q3.

The RFMS signal is linear with concentration up to 2.5 µM ($R^2 > 0.99$) for both PS and PE, which is below the upper limit of the PS or PE concentration (200 nM) in the final analysis samples. Experiments were run in triplicate and performed twice with equivalent results. Serial dilutions of a proprietary clean-up cartridge (BioTrove compound B) to remove non-volatile buffer and membrane components with H₂O in a 2.5 s wash cycle. The substrate and product were co-eluted to the mass spectrometer in 3.5 s using 80% isopropanol. The entire injection cycle was approximately 7.5 s per well. The chromatography system produced baseline-resolved peaks with widths of approximately 3 s.



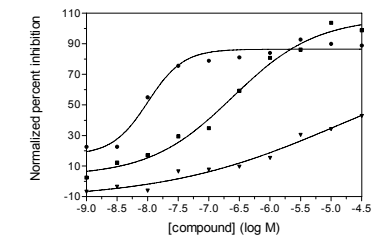
PSID in Automated Liquid Handling Mode

Samples contained PSID at 15, 30 or 45 µg/ml or a mitochondrial preparation from untransfected cells as a negative control at the same concentrations (only the 15 µg/ml control data is shown for clarity). The PSID prep shows linear kinetics, and the negative controls exhibit no activity. At 60 min, there is good separation of the signal distribution of the active and inactivated enzyme signals (error bars are within the size of the plot symbols). The apparent percent conversion at time zero reflects the presence of endogenous PE.



Representative IC₅₀ Experiments

Representative dose-response curves confirming activity of three hits of varying potency (10 nM, 0.25 µM and 5 µM respectively) identified in the primary screen.



Why HTS by Mass Spectrometry?

• Mass spectrometry is label free

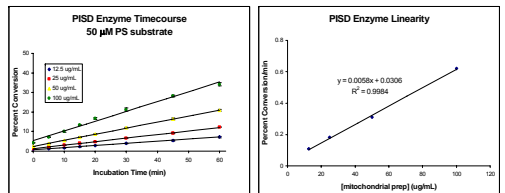
- overcomes limitation of traditional HTS formats by providing an alternative HTS approach to targets that typically would require

- i) fluorescent substrates/probes
- ii) radiolabeled substrates/probes
- iii) coupled assays

• Mass spectrometry can access intractable targets providing portfolio diversity via target novelty

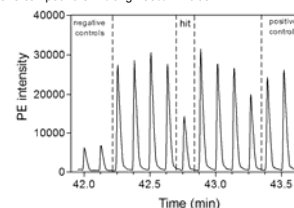
Enzyme Time Course

Product formation is linear with time as the PSID concentration is varied ($R^2 > 0.99$). Endogenous PE contributes to an apparent conversion at zero time, which is not significant at the screening concentration for PSID of 25 µg/ml. Product formation is linear with enzyme concentration ($R^2 > 0.99$). Samples contained 12.5, 25, 50 or 100 µg/ml PSID and 50 µM PS.



Representative raw HTMS Screening Data

The intensity of the PE mass spectrometry signal is shown with time from a series of wells within a plate chosen to show controls and a hit. Each peak is derived from a single well, with a well sampling time of approximately 7.5 s. The first and last pairs of peaks correspond to negative controls (boiled enzyme) and positive (no inhibitor) controls, respectively. The subsequent peaks correspond to samples containing test compounds of varying inhibition potency, with one compound exhibiting >50% inhibition.



Discussion

Most traditional HTS formats rely on either a label and/or capture technology to quantify substrate turnover and product formation. While established HTS formats such as SPA and FRET provide robust approaches to many target classes, there are enzymes that are intractable to conventional HTS formats due to the lack of a suitable labeling or capture technology because of synthetic limitations or economic constraints. Mass spectrometry provides a general, label-free approach to quantifying enzyme reactions but lacks the throughput requirements for HTS. The RapidFire™ high-throughput mass spectrometry platform allows for fast clean-up of screening samples which has been the traditional bottleneck to the use of mass spectrometry in HTS applications.

The method allows for PSID assays to be performed with robotic liquid handling and analyzed with mass spectrometry with a sample analysis time of 7.5 s. This allows for moderately rapid screening of compound libraries, although not with the throughput of traditional HTS formats. A primary screen of 9,920 compounds had an average Z' of 0.79 and 54 of 60 hits selected for confirmation were active in dose-response studies (IC₅₀ <15 µM). The assay is therefore robust and reliably identifies PSID inhibitors.

In conclusion, a high-throughput label-free mass spectrometry assay for screening PSID has been developed. The application of high-throughput mass spectrometry, therefore, permitted a high-quality screen to be performed for an otherwise intractable target.