

Identification of Fatty Acid Amide Hydrolase Inhibitors by a High Throughput Mass Spectrometry-based Assay

Tracy Chen¹, Richard Nugent¹, Can Özbal², William LaMarr², Jason Hughes¹, David Dudley¹, Kay Ahn¹, Ben Cravatt¹, and Norman Garceau¹

¹Pfizer Research Technology Center, 620 Memorial Drive, Cambridge, MA 02139, USA, ²BioTrove Inc., 12 Gill Street, Suite 4000, Woburn MA 01801, USA

Abstract

Here we describe a novel high throughput mass spectrometry (HTMS) assay that was developed to measure the activity of native and recombinant Fatty Acid Amide Hydrolase (FAAH). The HTMS assay quantifies the amount of arachidonic acid produced by the hydrolysis 2-arachidonylethanolamine (anandamide), one of several endocannabinoid substrates of FAAH. The data generated with the HTMS assay was consistent with data generated in-house utilizing a published high performance lipid chromatography (HPLC) assay. The enzyme kinetics for anandamide and pharmacological profile for reference inhibitors were consistent between the two assays and with published data. The HTMS assay was used to screen approximately 1.2 million compounds in an HTS campaign and several potential lead series were identified and characterized. Acyl azole was among the most potent series with IC₅₀ values ranging from 0.3nM to 2.4μM by HTMS method and their activities were confirmed in a cell-based assay. The kinetic characterization and mechanism of inhibition was further studied. This series was found to be irreversible inhibitors and evidence strongly suggested covalent modification of the tryptic peptide containing the S241 nucleophile with the added mass being 71 mass units. The HTMS method described in this study could be a valuable tool for screening other enzyme targets as well.

HTMS Method

Both anandamide and arachidonic acid can be quantitatively analyzed by electrospray ionization mass spectrometry (ESI-MS). The conversion of the substrate to product can be monitored by determining the concentration of either analyte at the end of the reaction. Since the anandamide substrate is best suited for positive ion ESI-MS while the arachidonic acid product can only be detected in negative ion ESI-MS, only the arachidonic acid product was monitored during high-throughput screening to avoid rapid mode switching in the mass spectrometer. To normalize the data an internal standard consisting of a stable isotope of arachidonic acid in which 8 hydrogens were substituted for 8 deuteriums was added to the reaction along with the quench solution. Assay development was performed on a Sciex API4000 triple quadrupole mass spectrometer interfaced to an Agilent 1100 HPLC. The limit of quantitation for arachidonic acid was below 100 nM and the dose-response was linear up to 10 μM.

Assay development

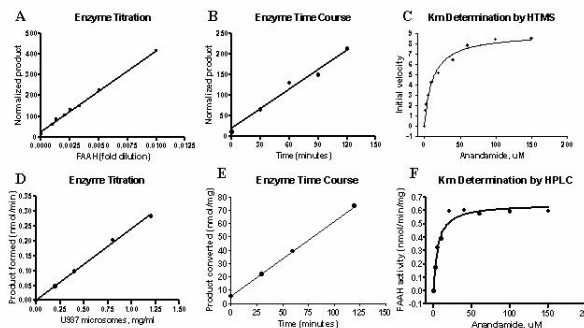


FIG. 1. Characterization of recombinant and endogenous FAAH from U937 microsomes by HTMS and HPLC. A, B, C represents data from recombinant FAAH and by HTMS method. D, E, F represents data from U937 microsomes and by HPLC method. A. Enzyme titration with 10μM anandamide and 1 hour incubation at 37°C. B. Enzyme time course with 400 fold dilution of FAAH and 10μM of anandamide. C. Km determination with 400 fold dilution of FAAH and 1 hour incubation at 37°C. D. Enzyme titration with 100μM anandamide and 75 minutes incubation at 37°C. E. Enzyme time course with 0.3mg/ml protein and 100μM anandamide. F. Km determination with 0.3mg/ml protein and 75 minutes incubation at 37°C.

Comparison of Enzyme Kinetic Data from Different Methods and Forms of the Enzyme

| Enzyme | *U937 microsomes | HPLC U937 microsomes | Rat liver microsomes | HTMS Human recombinant |
|-------------------|------------------|----------------------|----------------------|------------------------|
| Km (μM) | 3.6 | 5.2 | 6.5 | 11.5 |
| Vmax(nmol/mg/min) | 51.7 | 0.65 | 0.52 | 291 |

* Published data

HTS statistics

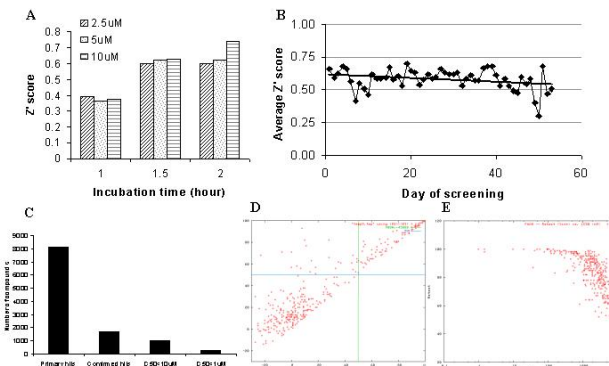


FIG. 3. HTS statistics. A. Z' score improvement. Increasing concentrations of substrate (2.5, 5 and 10μM) and incubation time (1, 1.5 and 2 hours) were used to improve the Z score. B. Average daily Z score during the entire screening. C. Number of primary hits, confirmed hits, and compounds that have IC₅₀ value under 10 or 1μM. D. Data reproducibility in a typical retest plate. Each axis represents percent inhibition from each run. E. Correlation between the IC₅₀ values (nM) and their percent inhibition.

Acyl azole series

| Compound | Structure | HTMS | Cell-based |
|----------|-----------|--------|------------|
| 1 | | 0.0003 | 0.001 |
| 2 | | 0.0003 | 0.001 |
| 3 | | 0.004 | 0.002 |
| 4 | | 0.006 | 0.006 |
| 5 | | 0.017 | 0.016 |
| 6 | | 0.018 | 0.007 |
| 7 | | 0.025 | 0.002 |
| 8 | | 0.028 | 0.098 |
| 9 | | 1.897 | 0.073 |
| 10 | | 2.44 | 0.063 |

Kinetic characterization

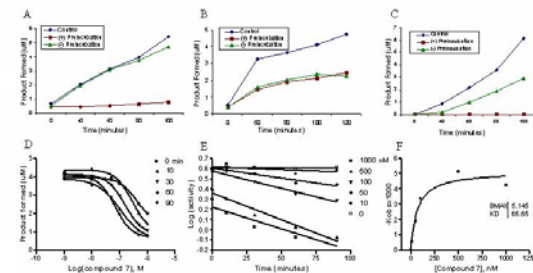


FIG. 4. Kinetic Characterization of the Acyl Azole Series. A. URB524 was used as an irreversible inhibitor control. B. PHOP was used as a reversible inhibitor control. C. Compound 7 from the new series was an irreversible inhibitor. D, E, F. represents data from compound 7. D. IC₅₀ curves shifted with different pre-incubation time. E. The logarithm of enzyme activity was linear with pre-incubation time. F. Ki determination via Michaelis-Menten equation.

Mechanism of inhibition

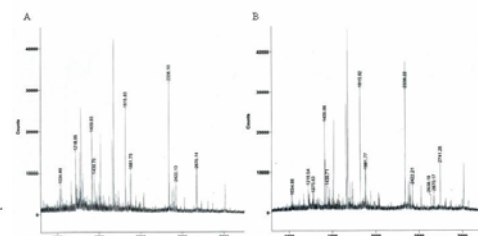


FIG. 5. The mechanism of inhibition of the acyl azole series by MALDI analysis. 10nM FAAH was treated with A. DMSO control. B. 200nM compound 7 for 1 hour at RT, and then digested by trypsin overnight at 37°C. Samples were processed and analyzed by MALDI-TOF Reflectron Spectrometry.