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

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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 of 2-arachidonoyl ethanolamine (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 0.3 nM to 2.4 µM 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.

Acyl azole series

Mechanism of inhibition

FIG 1. Characterization of recombinant and endogenous FAAH from U937 microsomes by HTMS and HPLC. A, B, C represent data from recombinant FAAH and by HTMS method. D, E, F represents data from U937 microsomes and by HPLC method. A. Enzyme titration with 100 mM arachidonic acid and 1 hour incubation at 37°C. B. Enzyme time course with 400 fold dilution of FAAH and 100 nM of arachidonic acid. C. Lin-log transformation with 400 fold dilution of FAAH and 1 hour incubation at 37°C. D. Enzyme titration with 200 µM anandamide and 75 minutes incubation at 37°C. E. Enzyme time course with 0.3 mg/ml protein and 100 µM anandamide. F. Lin-log transformation with 0.2 mg/ml protein and 75 minutes incubation at 37°C.

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

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>U937 microsomes</th>
<th>Rat liver microsomes</th>
<th>Human recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin (nM)</td>
<td>3.6</td>
<td>9.2</td>
<td>6.8</td>
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<tr>
<td>% Inhib.</td>
<td>51.7</td>
<td>63.5</td>
<td>52.2</td>
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<td>% Inhib.</td>
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</table>

* Published data

HTS statistics

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

Mechanism of inhibition

FIG 3. Mechanism of inhibition of the acyl azole series by mass spectrometry. A. DSIP was used as an irreversible inhibitor control. B. 7-HB was used as a reversible inhibitor control. C. Compound 7 from the new series was a reversible inhibitor. D, E, F represents data from compound 7. D. IC50 curves shifted, with different pre-incubation time. E. The logphase of enzyme activity was linear with pre-incubation time. F. IC50 determination via Michaelis-Menten equation.

Kinetic characterization

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