Application Note

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Discovery of Potential Soluble Epoxide Hydrolase Inhibitors Using a High-Throughput Screening Assay on a 235 Compound Library

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Key features

- Soluble epoxide hydrolase (sEH) inhibitors are a promising strategy for the treatment of inflammatory and cardiovascular conditions.
- An automated high-throughput screening (HTS) platform has been developed to identify small molecule inhibitors that enables testing of a large number of compounds in days instead of weeks using minimal sample volumes.
- All 235 compounds in Cayman's Anti-Inflammatory Screening Library were screened and 36 compounds were identified as potential sEH inhibitors using this approach.
- This HTS platform accelerates drug discovery. It can be adapted to many other enzyme activity assays and performed using Cayman or other compound libraries.

Introduction

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP450) metabolites of arachidonic acid formed by CYP450 epoxygenases and include four regioisomers: 5(6)-, 8(9)-, 11(12)-, and 14(15)-EET.[1] EETs have been shown to have a wide range of anti-inflammatory and cardioprotective effects.[2] However, EETs are rapidly hydrolyzed by soluble epoxide hydrolase (sEH) to their respective dihydroxyeicosatrienoic acids (DiHETrEs), a conversion that is coincident with significant loss of their biological activity. Accordingly, the use of sEH inhibitors to increase EET accumulation is a promising strategy for the treatment of inflammatory and cardiovascular conditions.

In this application note, a high-throughput screen (HTS) is described using a modified version of Cayman's Soluble Epoxide Hydrolase Inhibitor Screening Assay Kit adapted for automation and increased throughput. By automating this assay, data that would normally take 1 to 2 weeks to generate and analyze are obtained over the course of three days. Additionally, the use of automation drastically improves reproducibility and reduces both inter- and intra-assay variability. For this screen, Cayman's Anti-Inflammatory Screening Library, which consists of 235 biologically active and structurally diverse anti-inflammatory compounds, was evaluated. This screening library was chosen due to the relevance of sEH as a therapeutic target for inflammation.

Methods

Compound library and controls

An HTS of sEH inhibitors was performed in the HTS facility at Cayman Chemical. Cayman's Anti-Inflammatory Screening Library contains 235 compounds supplied as 10 mM stock solutions in DMSO in three 96-well tube racks. Compounds were reformatted to a single, mapped 384-well source plate for HTS. From the source plate, seven barcode-labeled dilution plates were produced, each containing vehicle, positive control, and 10 pt ½ log concentration-response curves for 32 compounds. An additional dilution plate was prepared as described above with 16 compounds. DMSO was used as the vehicle (negative) control, and the sEH inhibitor AUDA was used as the positive control.

Automated HTS assays

A fully automated platform consisting of three robotic instruments was used for this HTS assay (Figure 1). All liquid handling steps were performed using the Agilent Bravo automated liquid handling platform equipped with a 384 ST pipetting head. Assay and dilution plates were stored in separate stackers and placed on the pipetting deck of the Bravo using the Agilent BenchCel 4R microplate handler as required. Assay plates were transferred from the deck of the Bravo to the stage of an Agilent BioTek Synergy Neo2 hybrid multimode reader for data acquisition.

Test compounds were assayed in duplicate in black, barcode-labeled 384-well nonbinding plates. First, human recombinant sEH was diluted in sEH assay buffer, and 39.5 µL of this solution was transferred to each well of the assay plate. Next, 0.5 µL of test compound was transferred from the dilution plate to the assay plate. The reaction components were preincubated for 5 minutes at room temperature.

Enzymatic reactions were initiated by the addition of 10 µL of the sEH substrate PHOME. Assay plates were placed immediately on a Synergy Neo2 multimode microplate reader set to 25 °C using the BenchCel 4R microplate handler. Kinetic measurements were obtained every 3 minutes for a total of 20 minutes using a 360 nm excitation filter and 460 nm emission filter (360 excitation/460 emission). Following completion, assay and dilution plates were stored in separate stackers. Enzyme and substrate stocks, as well as inhibitor and substrate pipet tips, were refreshed between plates.

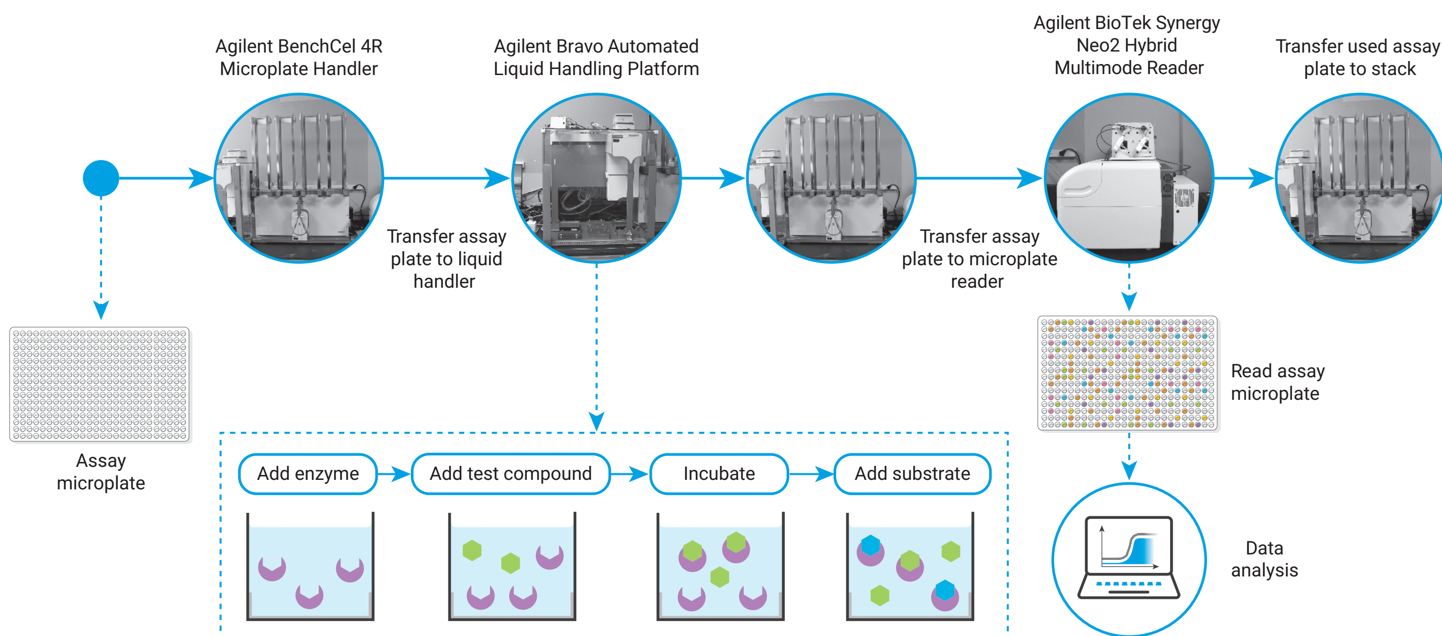


Figure 1. HTS assay workflow and reaction conditions. The Agilent Bravo automated liquid handling platform performs all liquid transfer steps in the HTS assay. The platform is integrated with an Agilent BenchCel 4R microplate handler to enable automated microplate handling to an Agilent BioTek Synergy Neo2 hybrid multimode reader.

Quality control

The Z'-factor is a widely used metric to evaluate an assay's suitability for HTS applications.[3] It measures assay robustness by determining the separation between the positive and negative controls. The Z'-factor was calculated using the following equation:

Equation 1.

$$Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where σ_p is the standard deviation of the positive control (AUDA), σ_n is the standard deviation of the negative control (DMSO), μ_p is the mean of the positive control, and μ_n is the mean of the negative control. Assays with Z'-factors between 0.5 and 1 are considered excellent assays.

Data analysis

Enzymatic rates were determined by calculating the increase in fluorescence versus time from the slope of the linear portion of the reaction. Maximum fluorescence was obtained at 20 minutes.

Percent activity values for test compounds were calculated based on wells containing the vehicle control (100% activity) and wells containing the positive control (sEH inhibitor AUDA). IC_{50} values were calculated using GraphPad Prism with a four-parameter non-linear regression curve fit.

Some compounds exhibited background fluorescent properties at high concentrations. This was determined by plotting compound fluorescence at the start of the assay versus compound concentration. Data were omitted in instances where compound fluorescence caused significant interference with the assay or data analysis. Data were retained in instances where compound background fluorescence caused no significant impact to the sEH inhibitory rate.

Assay performance

Representative vehicle and positive control responses are shown in Figure 2. The average Z'-factor for all assays was 0.60, indicating robust assay performance. A throughput of 20 compounds/hour was achieved using the automated HTS method described in the Methods section (data not shown).

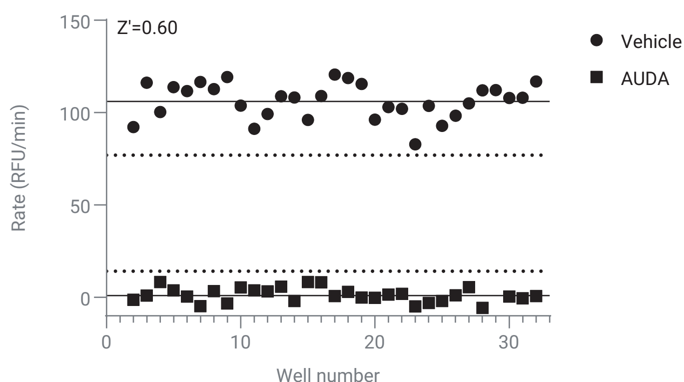


Figure 2. Scatterplot showing the vehicle control (solid circles; DMSO) and positive control (solid squares; AUDA) samples for the Z'-factor calculation of a representative 384-well plate in the sEH inhibitor HTS assay. Each point represents an individual well on a single plate (n = 32 for each control). The solid lines represent the mean fluorescence rates of the positive and negative controls. The dashed lines indicate 3 standard deviations above or below the mean. The Z'-factor calculated for this was data was 0.60, indicating excellent assay performance.

Results and discussion

IC_{50} values for sEH inhibition obtained in the HTS assay are shown for all 235 anti-inflammatory compounds in Figure 3. An IC_{50} value of 10 µM was used as the baseline value to compare all compounds. IC_{50} values below 10 µM were considered potential sEH inhibitors whereas compounds with IC_{50} values above 10 µM were considered unlikely sEH inhibitors. 10 µM was chosen to capture weaker secondary target activity, as would be anticipated with compounds with known primary targets. In total, 36 anti-inflammatory compounds were discovered with sEH IC_{50} values below 10 µM in this HTS.

Six hit compounds that exhibited potent IC_{50} values and/or clear concentration-response curves are shown in Figure 4. The main anti-inflammatory targets, primary target potency, and sEH IC_{50} values of these six hit compounds are summarized in Table 1. SC-75741, an NF-κB inhibitor, and *trans*-AUCB, a known sEH inhibitor, were identified as potent sEH inhibitors in this HTS assay.[4,5] Both compounds inhibited sEH with potency that exceeded the range of concentrations tested (10 nM to 10 µM). Therefore, IC_{50} values for these compounds are indicated as <10 nM. Given these potencies, these compounds are prime candidates for secondary screening.

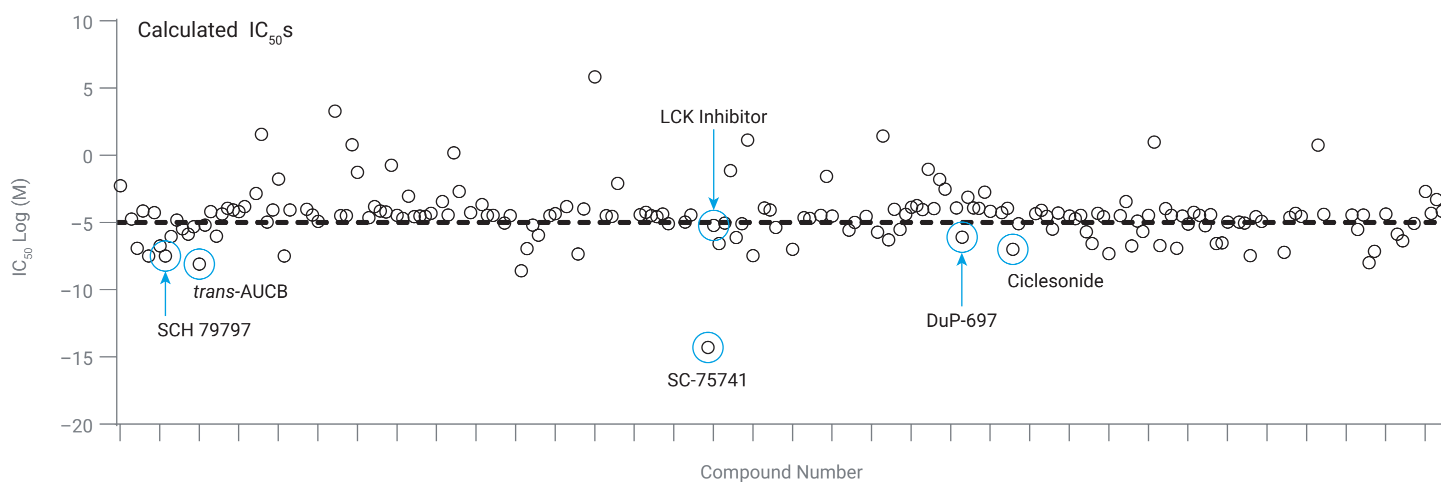
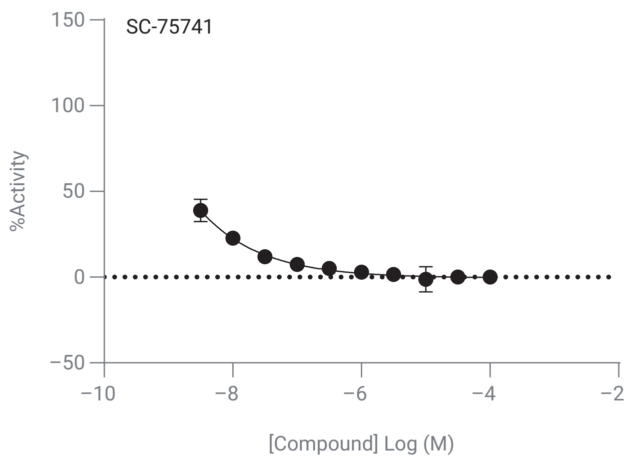
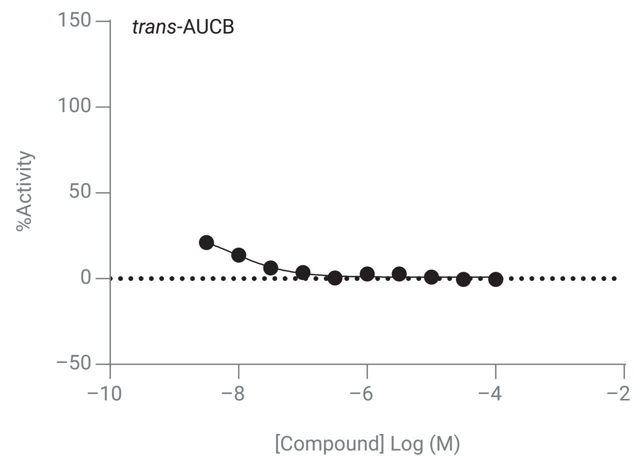
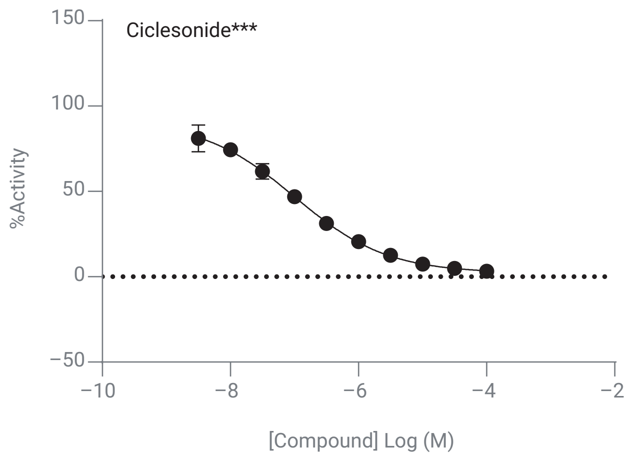
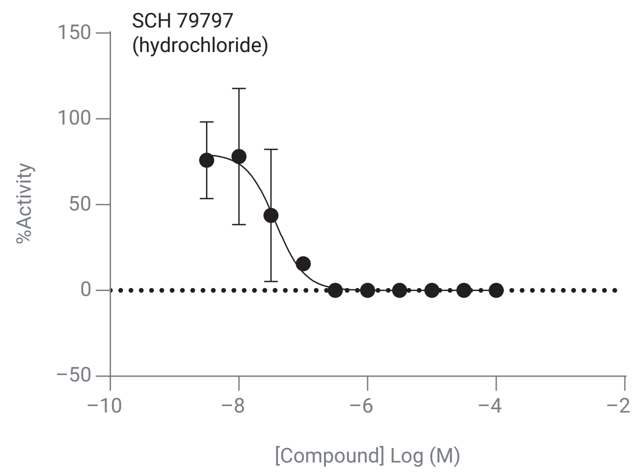


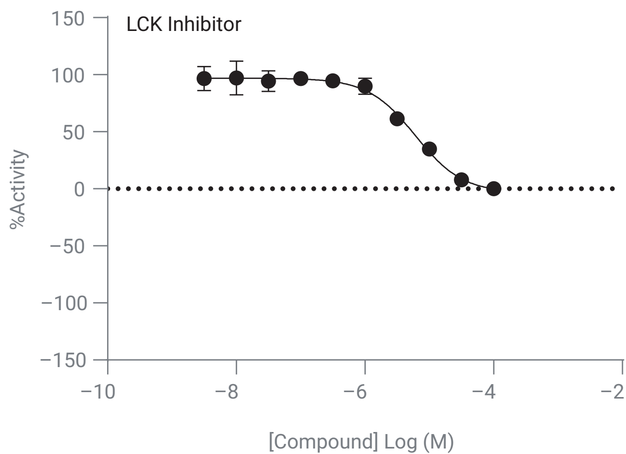
Figure 3. Scatterplot of IC_{50} values for test compounds. The baseline value used to compare all compounds was 10 µM (−5 Log (M)). Compounds with IC_{50} values less than 10 µM were considered potential sEH inhibitors, whereas compounds with IC_{50} values greater than 10 µM were considered unlikely inhibitors. Compounds circled in red exhibited potent IC_{50} values and/or clear concentration-response curves.











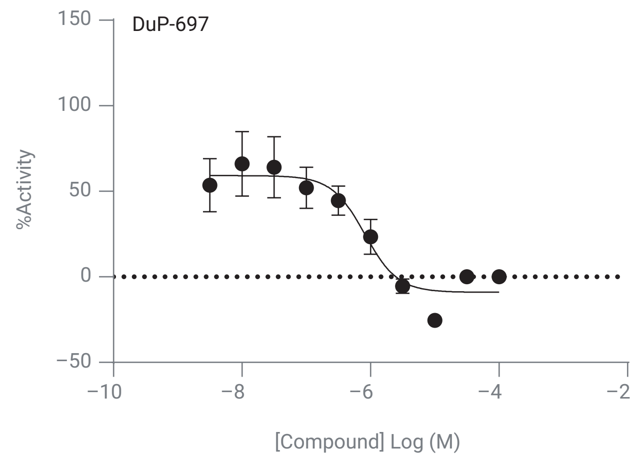


Figure 4. Concentration-response curves of select hit compounds. All data are plotted as mean ± standard deviation of two technical replicates. The Z'-factor values for assay plates containing these compounds were >0.5. Compounds marked with a triple asterisk (***) exhibited background fluorescent properties at high concentrations.

Ciclesonide is a prodrug of the glucocorticoid receptor agonist desisobutyryl ciclesonide.[6] Glucocorticoids are amongst the most well-known anti-inflammatory compounds. Despite ciclesonide displaying background fluorescence at high concentrations, it was determined that ciclesonide has an IC_{50} value of 100 nM for the inhibition of sEH. Ciclesonide has limited solubility in aqueous solutions. The higher compound concentrations used in this HTS assay may have approached the limit of its aqueous solubility, which could have contributed to high background fluorescence.

SCH 79797, an antagonist of proteinase-activated receptor 1 (PAR1), was found to have sEH inhibitory activity in this screen (IC_{50} = 372 nM).[7] SCH 79797 inhibits PAR1 activation on vascular smooth muscle cells and endothelial cells as well as α-thrombin-induced platelet aggregation. Whether or not the sEH inhibitory activity of SCH 79797 contributes in part to these cardioprotective effects could be explored in follow-up studies.

DuP-697, a selective COX-2 inhibitor, was discovered to be a potential sEH inhibitor (IC_{50} = 813 nM).[8] This finding is especially intriguing given the extensive role of COX-2 in inflammation. COX-2 is primarily responsible for the production of prostaglandins (PGs) from arachidonic acid, a family of lipid signaling molecules with multiple inflammatory functions. Using dual inhibitors of COX-2 and sEH, potentially like DuP-697, to halt PG production concurrent with increasing tissue EET accumulation is an intriguing approach for the treatment of inflammatory conditions.

LCK Inhibitor inhibits the Src-family non-receptor protein tyrosine kinase LCK.[9,10] LCK is expressed by T cells and mediates IL-2 production, a hallmark of T cell activation, in response to T cell receptor signaling. Here, it was discovered that LCK Inhibitor also inhibits sEH activity (IC_{50} = 6,070 nM). Thus, it could be of interest for future studies to determine if LCK Inhibitor increases EET levels and if this is a viable strategy to dampen aberrant T cell activity.

Table 1. Comparison of IC_{50} values for primary target and sEH of six hit compounds identified in this automated HTS assay.

Compound	Primary Target - Target Potency (IC_{50})*	sEH (IC_{50})
SC-75741	NF-κB - 200 nM[4]	<10 nM
trans-AUCB	sEH - 0.5 nM[5]	<10 nM
Ciclesonide	Glucocorticoid receptor - K_i = 37 nM[6]	100 nM
SCH 79797	PAR1 - 70 nM[7]	372 nM
DuP-697	COX-2 - 80 nM[11]	813 nM
LCK Inhibitor	LCK - <1-2 nM[9,10]	6,070 nM

* Data are shown for IC_{50} values unless otherwise indicated.

Conclusion

This application note established an automated HTS platform to identify small molecule inhibitors. This automated HTS assay is an efficient way to screen compound libraries against a specific target. Given the throughput, this assay could easily be applied to a larger compound library to increase the probability for hits. With this automated format, multiple assays can be easily modified to encompass a wide range of target enzymes and compound library combinations. This automated HTS assay is applicable to basic research as well as drug discovery.

About the authors

David received his PhD in 2009 from the University of Rochester. After the completion of a post-doc, he left academia to join a small drug discovery company in 2011. He joined Cayman Chemical in 2013 as an R&D scientist where he aided in the development of numerous kits targeted at mitochondrial function. In 2018, he started working to build Cayman's cellular metabolism/high throughput screening services group, and is currently serving as Scientific Director for the Division.

Melissa received her PhD in 2017 from Michigan State University. After a post-doc, she joined Cayman Chemical in 2019 as a Technical Writer and moved into Marketing as a Scientific Content Developer in 2022.

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