Fürster resonance energy transfer competitive displacement assay for human soluble epoxide hydrolase

Kin Sing Stephen Lee, Christophe Morisseau, Jun Yang, Peng Wang, Sung Hee Hwang, Bruce D. Hammock

A R T I C L E   I N F O

Article history:
Available online 5 December 2012

Keywords:
Soluble epoxide hydrolase
Binding assay
Competitive displacement assay
Fürster resonance energy transfer (FRET)
Fluorescent assay

A B S T R A C T

The soluble epoxide hydrolase (sEH), responsible for the hydrolysis of various fatty acid epoxides to their corresponding 1,2-diols, is becoming an attractive pharmaceutical target. These fatty acid epoxides, particularly epoxyeicosatrienoic acids (EETs), play an important role in human homeostatic and inflammation processes. Therefore, inhibition of human sEH, which stabilizes EETs in vivo, brings several beneficial effects to human health. Although there are several catalytic assays available to determine the potency of sEH inhibitors, measuring the in vitro inhibition constant ($K_i$) for these inhibitors using catalytic assay is laborious. In addition, $K_i$, which has been recently suggested to correlate better with the in vivo potency of inhibitors, has never been measured for sEH inhibitors. To better measure the potency of sEH inhibitors, a reporting ligand, 1-(adamantan-1-yl)-3-(1-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetyl)piperidin-4-yl)urea (ACPU), was designed and synthesized. With ACPU, we have developed a Fürster resonance energy transfer (FRET)-based competitive displacement assay using intrinsic tryptophan fluorescence from sEH. In addition, the resulting assay allows us to measure the $K_i$ values of very potent compounds to the picomolar level and to obtain relative $k_{cat}$ values of the inhibitors. This assay provides additional data to evaluate the potency of sEH inhibitors.

© 2012 Elsevier Inc. All rights reserved.
concentration of enzyme is needed (50–100 nM), making this assay costly. In addition, several recent inhibitors developed by several groups have nanomolar or sub-picomolar half-maximal inhibitory concentration (IC\textsubscript{50}) values. Because the binding probes used in the above assay do not bind the sEH tightly (IC\textsubscript{50} ~1 μM), it is not suitable to measure the binding of very potent inhibitors with \( K_i \) values lower than 10 nM [16].

With some inhibitors, such as cyclooxygenase inhibitors, residency time on the enzyme correlates better with in vivo efficacy than does enzyme inhibition potency [22]. Thus, an assay that could estimate occupancy time of the sEH inhibitors will be an invaluable tool to provide another dimension to estimate the in vivo potency of these inhibitors. However, none of the described assays is able to measure the \( k_{off} \) of the inhibitor directly [13]. Here, we report a newly developed Förster resonance energy transfer (FRET)-based displacement assay using fluorescence from intrinsic tryptophan to measure the binding affinity (\( K_i \)) and relative \( k_{off} \) of very potent inhibitors of sEH.

**Materials and methods**

**Chemicals**

All reagents and solvents were purchased from Fisher Scientific, Acros Organics, TCI America Fine Chemicals, and Sigma–Aldrich and were used directly without further purification. 14,15-Epoxyecosatrienoic acid (14,15-EET) was purchased from Cayman Chemicals. The syntheses of tert-butyl 4-[(3-adamantan-1-yl)ureido]piperidine-1-carboxylate, tert-butyl 4-[(3-(4-fluoromethyl)phenyl)ureido] piperidine-1-carboxylate, 1-(adamantan-1-yl)-3-(piperidin-4-yl)urea, 1-(piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea, 1-(adamantan-1-yl)-3-(piperidin-4-yl)ureido) piperidine-1-carboxylate, 1-(adamantan-1-yl)-3-(4-(trifluoromethyl)phenyl)urea (PTU), and 1-[(1r,4r)-4-hydroxycyclohexyl]-3-(4-(trifluoromethoxy)phenyl)urea have been reported elsewhere [5, 21, 23]. All reactions for this study were carried out in a dry nitrogen atmosphere. 19F NMR spectra were recorded on a Varian QE-300 spectrometer at 75 MHz. 13C NMR spectra were recorded on a Varian QE-300 spectrometer at 75 MHz. 1H NMR (300 MHz, DMSO-\( \text{d}_6 \), 300 MHz): 8.78 (s, 1H), 7.57 (s, 4H), 6.39 (d, \( J = 7.5 \) Hz, 1H), 3.61 (m, 1H), 3.46 (d, \( J = 12.3 \) Hz, 2H), 2.87 (s, 3H), 2.87 (m, 2H), 1.92 (d, \( J = 9.9 \) Hz, 2H), 1.46 (m, 2H).

### Synthesis of 1-(1-(2-methylbutyryl)piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea (11)

2-Methylbutyric acid (50 mg, 0.487 mmol), DMAP (54.5 mg, 0.487 mmol), and EDCI (64 mg, 0.325 mmol) were dissolved in dimethylformamide (DMF, 10 ml). PTU (55 mg, 0.325 mmol) was dissolved in DMF (5 ml) and was added into the reaction mixture dropwise. The reaction mixture was stirred for 12 h at rt and was quenched by the addition of HCl solution (1 M, aq). The organic layer was collected, and the aqueous layer was extracted with EtOAc four times. The combined organic layer was concentrated in vacuo and further purified by flash chromatography (EtOAc/Hex, 2:1), yielding the final product (80 mg, 0.215 mmol, 66% yield).

### Synthesis of 1-(1-(methanesulfonyl)piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea (12)

PTU (70 mg, 0.244 mmol) and Et\textsubscript{3}N (30 mg, 0.292 mmol) were dissolved in DMF (10 ml) at 0 °C, and methylsulfonyl chloride (56 mg, 0.487 mmol) was added into the reaction mixture dropwise. The reaction mixture was stirred for 12 h at rt and was quenched by the addition of HCl solution (1 M). The organic layers were collected, and the aqueous layer was extracted with EtOAc four times. The combined organic layers were concentrated in vacuo and further purified by flash column chromatography (EtOAc/Hex, 6:4), yielding the final product (45 mg, 0.123 mmol, 51% yield).

### Synthesis of 1-(1-(butane-1-sulfonyl)piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea (13)

Butanesulfonyl chloride (76 mg, 0.487 mmol) was reacted with PTU (70 mg, 0.244 mmol) in the same manner as the synthesis of 1-(1-(methylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea, yielding the final product (56 mg, 0.160 mmol, 66% yield).

### Enzyme preparation

Expression and purification of recombinant sEH followed the published procedure [25]. Briefly, the full-length human complementary DNA (cDNA) for sEH was expressed in high yield in a baculovirus system. The sEH activity in supernatant from cell culture was purified by affinity chromatography to yield high specific activity and apparent homogeneity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see Fig. S1 in Supplementary material). The enzyme was frozen in multiple small aliquots and thawed once immediately before use.

**IC\textsubscript{50} determination for hsEH inhibitors**

IC\textsubscript{50} values of human sEH (hsEH) inhibitors were determined by three different assays (radiometric, fluorescent, and liquid chroma-
tography–tandem mass spectrometry (LC–MS/MS) according to published procedures [14–18]. The assays are described in detail in the Supplementary material. IC50 values were determined based on regression of at least five data points with a minimum of two points in the linear region of the curve on either side of the IC50.

**Fluorescence binding assay procedure**

The fluorescent binding assay was carried out based on the published procedure with some modifications as described below [15]. All inhibitors were stored in glass containers to prevent them from being absorbed by plastic. Some plastic containers were found to leach fluorescent impurities into sample, which affects the fluorescent analysis. sEH was diluted in filtered sodium phosphate buffer (PB buffer, 100 mM, pH 7.4) with 0.01% gelatin (Sigma–Aldrich) to prevent the loss of sEH due to nonspecific binding to the surface of the cuvette. sEH (10 nM, 3 ml in phosphate buffer with 0.01% gelatin, pH 7.4) was added into the cuvette and was stirred gently. The solution was equilibrated at 30 °C for 5 min. The sample was excited at 280 nm, which is the λmax of the protein (see Fig. S3c in Supplementary material) (excitation slit width = 1.0 nm), and the emission at the protein peak maximum (325–360 nm, emission slit width = 12 nm) (Fig. S3a) and the emission of coumarin (450 nm, emission slit width = 12 nm) (Fig. S3b) were measured. Reporting ligand dissolved in EtOH was added to the sample at varying concentrations (0.2–20 nM). The total concentration of EtOH was kept under 2%.

Because coumarin fluoresces on excitation at 283 nm and absorbs at 330 nm, which could quench the tryptophan fluorescence from the protein, a correction was made prior to calculating the binding constant for sEH with the reporting ligand. A blank titration with the reporting ligand (ACPU): the concentration of reporting ligand with 110 nM sEH, the free ligand concentration: (3) Estimate the fluorescence contribution of the free ligand, F0, to be deduced from the blank (N-acetyltryptophanamide) titration.

(4) Subtract the fluorescence contribution of the free ligand based on the actual readings and plot the corrected fluorescence values versus the ligand concentration: (F – F0) versus $R_i$.

The $K_d$ value for each sEH inhibitor was determined according to the method previously described by Wang and coworkers using the fluorescence values calculated above [28].

The dissociation constant is calculated by KaleidaGraph using nonlinear least squares regression analysis. The corrected relative fluorescence intensity is plotted against the concentration of each sEH inhibitor. The protein concentration and the final fluorescence intensity at saturation are determined in order for the program to calculate the dissociation constant:

$$\frac{F}{F_i} = 1 + \frac{F_0}{F_i} \times \left( \frac{P_t + R_t + K_d - \sqrt{(P_t + R_t + K_d)^2 - 4P_tR_t}}{2P_t} \right)$$

where $F$ is observed fluorescence, $F_i$ is fluorescence of free protein, $F_0$ is fluorescence of bound protein, $P_t$ is total protein concentration, and $R_t$ is total reporting ligand concentration.

**FRET displacement assay procedure**

As mentioned, to prevent leaching of fluorescent impurities from the plastic tube and nonspecific binding to sEH inhibitors, the inhibitor was stored in glass vials. In addition, sEH was dissolved in phosphate buffer (100 mM sodium phosphate, pH 7.4, 0.01% gelatin) to avoid loss of protein from nonspecific binding to the cuvette surface. sEH (10 nM, 3 ml, 100 mM phosphate, pH 7.4, 0.01% gelatin) was stirred and preincubated with reporting ligand (1 equivalent) at 30 °C for 1 h in a quartz cuvette. The fluorescence (455 nm, emission slit width = 12 nm, excited at 280 nm, excitation slit width = 1.0 nm) was measured. The enzyme–ligand complex was titrated with different sEH inhibitors at varying concentrations until no more fluorescence quenching was observed. The relative fluorescence intensity was plotted against the concentration of inhibitor.

**Curve fitting**

The data manipulation and $K_i$ calculation were based on the original article by Roehrl and coworkers [29,30].

The displacement assay has three components, two ligands and one protein, which creates three-state equilibrium binding models:

$$[R] + L \leftrightarrow R + I + L \leftrightarrow [R] + I \quad 4$$

The three-state equilibrium Eq. (4) is composed of the sEH–inhibitor complex, sEH, and the sEH–reporting ligand complex. The relative fluorescence intensity ($F_3$) was plotted against the concentration of sEH inhibitor, and the curve was fitted into Eq. (5) derived by Wang for three-state equilibrium [29]:

$$F_3 = \frac{2(a^2 - 3b)^{1/2} \cos(\theta/3) - a}{3K_{d1} + 2(a^2 - 3b)^{1/2}} \times \cos(\theta/3) - a$$

with $a = K_{d1} + K_{d2} + L + I - R$;

$b = K_{d2}(L - R) + K_{d1}(I - R) + K_{d1}K_{d2};$

$c = -K_{d1}K_{d2}R; \quad \text{and}$

$\theta = \arccos\left(\frac{-2a^2 + 9ab - 27c}{2(a^2 - 3b)^{3/2}}\right).$

where $F_3$ is relative fluorescence and is equal to (observed fluorescence – fluorescence at saturation)/(initiated fluorescence – fluores-
cence at saturation). \( I \) is concentration of added unlabeled competing ligand, \( R \) is total concentration of sEH, \( I \) is total concentration of reporting ligand, \( K_d \) is dissociation constant of reporting ligand (found by fluorescent binding assay), and \( K_{dR} \) is inhibition constant of inhibitors.

### \( k_{off} \) measurement procedure

sEH (8 \( \mu \)M) was incubated with inhibitor (8.8 \( \mu \)M, 100 mM PB buffer, pH 7.4) for 1.5 h at 30 °C. The sEH–inhibitor complex was diluted 40 times against 100 equivalents of ACPU (20 \( \mu \)M, 100 mM PB buffer, pH 7.4). The fluorescence at 450 nm (excited at 280 nm) was measured every 30 s for 5000 s. The fluorescence (emission at 450 nm) was plotted against time (s). The resulting curve was fitted to single exponential growth, and the relative \( k_{off} \) was obtained. This procedure was then applied to other inhibitors using the same procedure as described above for ACPU.

### Results and discussion

#### Design and synthesis of fluorescent reporter

hsEH is a predominantly cytoplasmic enzyme containing both epoxide hydrolase domain at the C terminus and phosphatase domain at the N terminus [31–33]. From the holo structure of hsEH with its inhibitor, \( N \)-cyclohexyl-\( N' \)-(4-iodophenyl)urea, 10 tryptophans are found on the C terminus near the catalytic cavity where the inhibitors bind (see Fig. S2 in Supplementary material) [32]. Such tryptophans, which absorb at 280 nm and fluoresce between 330 and 380 nm, are well-known intrinsic fluorophores that have been used routinely in biomedical and biochemical research to study protein–ligand or protein–protein interactions [26,27,34,35]. To develop a FRET displacement assay, we chose these intrinsic tryptophans as fluorescent donors, and a reporting ligand that contains the complementary fluorescent acceptors will be designed and synthesized.

Crystal structures showed that 3 tryptophans are located within the binding pocket. To position the fluorescent acceptor close to these tryptophans for maximizing FRET, a small fluorophore that can fit within the binding pocket is needed. In addition, a fluorophore whose absorption spectrum overlapped with the fluorescence of tryptophan is needed to enhance the energy transfer process. Besides, a fluorophore that has relatively high quantum yield can enhance FRET signal. Therefore, 7-hydroxycoumarin, which absorbs at 360 nm with a quantum yield around 0.6 [36], was chosen as the fluorescent acceptor for the reporting ligand (Fig. S3a). In addition, a relatively tight binding inhibitor scaffold is desired for the reporting ligand. From the large number of published structures of potent inhibitors [10], adamantane was selected as a functional group yielding highly potent inhibitors [5,21] and fitting tightly in the “small” binding pocket of the sEH enzyme while lacking an aromatic moiety that could quench the intrinsic tryptophan fluorescence of the sEH with a 300-nm broad absorbance band. For the “large” or right binding pocket on the 3 position of the urea, we selected a piperidyl group for the same reasons to give a 1-adamantyl-3-piperidyl scaffold. We wanted a reporting ligand with a low \( K_d \) to improve our ability to distinguish among inhibitors with \( K_i \) values in the nanomolar to picomolar (nM to pM) binding range [37]. Thus, our reporting ligand (ACPU), which contains one of the common hsEH inhibitor scaffolds, 1-adamantyl-3-piperidyl urea (A), and a fluorophore, coumarin (B), was designed and synthesized (Fig. 1A). Modeling of sEH with the reporting ligand suggests that ACPU should fit nicely inside the binding pocket, and 6 tryptophans are located within 12 Å of the coumarin (Fig. 1B).

### Measuring binding constant (\( K_d \)) for fluorescent reporter (ACPU)

To determine a near optimal incubation time for each titration, the rate of binding between ACPU and hsEH was studied based on the FRET signal at 450 nm. The results indicated that 40 min was needed to reach the equilibrium between ACPU and sEH under the conditions described (Fig. S4). Then, sEH was titrated with the ACPU to obtain the \( K_d \) of the reporting ligand. From the titration, we observed that the fluorescence at 348 nm from the protein decreased, whereas the fluorescence at 450 nm increased proportionally to the ACPU concentration, as would be expected from FRET (Fig. S5). Because the ACPU can also be excited at 280 nm, the fluorescence enhancement from the assay at 450 nm was corrected for background fluorescence at different concentrations before it was used to calculate the dissociation constant (\( K_d \)) of ACPU (see Materials and methods). Using the nonlinear regression analysis of the quadratic equation Eq. (3) derived from the thermodynamic and mass law [38], the \( K_d \) of ACPU for hsEH is calculated to be 1.04 ± 0.04 nM, indicating a tight binding ligand. This value is the same whether it is based on the fluorescence decrease at 340 nm or the fluorescence enhancement at 450 nm plotted against different concentrations of ACPU (Fig. 2). Compared with previous reporting ligands (\( K_d = 1 \mu \)M) [13,16], the high potency of ACPU permits one to measure the \( K_i \) of very tight binding inhibitors accurately [37,39].

#### Development and optimization of competitive displacement assay with ACPU

A competitive displacement assay based on the above-established FRET system was then developed to determine inhibition constants (\( K_i \)) for sEH inhibitors. Because the \( K_d \) of the ACPU is known, the \( K_i \) of the inhibitor can be calculated through measuring the fluorescence at 348 nm from the protein excited at 280 nm (Fig. S6) with different concentrations of inhibitors (Fig. 3A). In the presence of the inhibitor, the ACPU displacement from the binding pocket due to the competition of ACPU with the inhibitor is reflected by the decrease in fluorescence at 348 nm. The dissociation constant \( K_i \) of the inhibitor can be calculated through measuring the fluorescence at 348 nm from the protein excited at 280 nm with different concentrations of inhibitors (Fig. 3A). In the presence of the inhibitor, the ACPU displacement from the binding pocket due to the competition of ACPU with the inhibitor is reflected by the decrease in fluorescence at 348 nm. The dissociation constant \( K_i \) of the inhibitor can be calculated through measuring the fluorescence at 348 nm from the protein excited at 280 nm with different concentrations of inhibitors (Fig. 3A).

![Fig. 1](image-url)
the relative occupancy of sEH by ACPU through its FRET signal at 450 nm as a function of the concentration of inhibitor. Here, the FRET signal at 450 nm was chosen because the emission at 330 nm from the sEH could result from interference not only by inhibitors that have aromatic pharmacophores but also by the changes of protein environment when inhibitors bind [16, 35]. In addition, the FRET signal at 450 nm shows more significant change than the FRET signal at 330 nm (Fig. S5), which could help to improve the signal-to-noise ratio of the assay.

To determine the optimal temperature for the displacement assay, we investigated the rate of ACPU displacement by the sEH inhibitor, \( t\)-TUCB (16), at different temperatures. As expected, the rate for ACPU displacement by sEH inhibitors increases as a function of the temperature (Fig. S6). The time needed for reaching equilibrium on the addition of inhibitor decreased from 118 min at 25 °C to 75 min at 37 °C. Although increasing the temperature can shorten the time of reaching equilibrium by 15 min, the half-life of hsEH at 37 °C is 1.3 h, whereas at 30 °C it is 8.1 h [25]. Therefore, we decided to conduct the assay at 30 °C, and the incubation time for each titration was 90 min. (Only 5% of the fluorescence of the reporter-bound hsEH was diminished per hour [data not shown], indicating that the enzyme is stable throughout the displacement experiment [25].)

Through titration of the ACPU–hsEH complex with the sEH inhibitor, a decrease of fluorescence at 450 nm when excited at 280 nm was observed, indicating that the ACPU was displaced by the inhibitor (APAU, 2) (Fig. 3). By plotting the relative fluorescence against the concentration of inhibitor, we obtain a displacement curve that can be used to calculate \( K_i \) of the inhibitors based on the \( K_d \) of ACPU by fitting the curve to the Wang-derived equation Eq. (5) [29].

The classic approach for \( K_i \) calculation is based on the Scatchard plot. This approach, which is used by most of the curve fitting programs, assumes that less than 10% of added ligand is bound [40–42]. However, some of the sEH inhibitors we tested are very potent, with IC\(_{50}\) values below 1 nM. Therefore, these inhibitors would violate the assumptions associated with this classical approach. Wong reported a general equation Eq. (5) based on the three equilibrium states among inhibitor, ACPU, and targeted enzyme Eq. (4) to calculate the inhibition constant (\( K_i \)) of the inhibitor for competitive displacement assay. This approach provides a general equation to calculate the inhibition constant (\( K_i \)) from displacement assay without any assumptions [29]. The calculated \( K_i \) values of several compounds are shown in Table 1. In general, the \( K_i \) values determined with ACPU are lower than those obtained previously using trans-diphenyl-propene oxide (\( t\)-DPPO) in a catalytic assay. Besides being labor-intensive and expensive, this previously used assay could overestimate \( K_i \) values because a significant amount of the inhibitor was bound to the enzyme, which violates the assumption of the equation for \( K_i \) calculation [43].

To increase the throughput for displacement assay, it was also formatted for a 96-well plate. However, to obtain a fluorescence signal at 450 nm at least 5-fold above the background, 100 nM pure hsEH was needed (data not shown). The \( K_i \) values obtained from the 96-well plate assay correlated well with those determined by titration in a cuvette (Table 1). However, very potent compounds (\( K_i < 1 \) nM) need their inhibitory constant to be determined by titration in a cuvette because the nonlinear regression analysis can accurately calculate the \( K_i \) within two orders of magnitude of enzyme concentration [41].

**Comparison of potency of sEH inhibitors using different assays**

Several assays have been developed to measure the potency of sEH inhibitors since the first assay was reported; however, most of
<table>
<thead>
<tr>
<th>Inhibitor number (and name)</th>
<th>Reporter:</th>
<th>Structure</th>
<th>ACPU(e) (nM)</th>
<th>t-DPPO(a) (nM)</th>
<th>CMNPC(b) IC50 (nM)</th>
<th>EET(c) IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (AUDA)</td>
<td></td>
<td><img src="image1" alt="Structure" /></td>
<td>0.23 ± 0.07</td>
<td>0.11 ± 0.03</td>
<td>6.8 ± 0.2</td>
<td>14.8</td>
</tr>
<tr>
<td>2' (APAU)</td>
<td></td>
<td><img src="image2" alt="Structure" /></td>
<td>25.3</td>
<td>21.7 ± 3.10</td>
<td>125 ± 15</td>
<td>571</td>
</tr>
<tr>
<td>3 (APBU)</td>
<td></td>
<td><img src="image3" alt="Structure" /></td>
<td>2.62</td>
<td>3.02 ± 0.43</td>
<td>nd</td>
<td>10</td>
</tr>
<tr>
<td>4 (t-AUCB)</td>
<td></td>
<td><img src="image4" alt="Structure" /></td>
<td>2.73 ± 1.50</td>
<td>1.93 ± 0.09</td>
<td>1.5 ± 0.2</td>
<td>3.7</td>
</tr>
<tr>
<td>5 (t-TUCol)</td>
<td></td>
<td><img src="image5" alt="Structure" /></td>
<td>11.3</td>
<td>8.60 ± 0.57</td>
<td>nd</td>
<td>131</td>
</tr>
<tr>
<td>6 (t-AUCM)</td>
<td></td>
<td><img src="image6" alt="Structure" /></td>
<td>3.07</td>
<td>2.75 ± 0.72</td>
<td>nd</td>
<td>43</td>
</tr>
<tr>
<td>7 (t-TUCone)</td>
<td></td>
<td><img src="image7" alt="Structure" /></td>
<td>25.2</td>
<td>nd</td>
<td>nd</td>
<td>667</td>
</tr>
<tr>
<td>8 (TmPAU)</td>
<td></td>
<td><img src="image8" alt="Structure" /></td>
<td>14.8</td>
<td>nd</td>
<td>nd</td>
<td>511</td>
</tr>
<tr>
<td>9 (TPCU)</td>
<td></td>
<td><img src="image9" alt="Structure" /></td>
<td>0.67</td>
<td>nd</td>
<td>nd</td>
<td>20</td>
</tr>
<tr>
<td>10 (TmPPU)</td>
<td></td>
<td><img src="image10" alt="Structure" /></td>
<td>Nd</td>
<td>2.96 ± 0.60</td>
<td>nd</td>
<td>48</td>
</tr>
<tr>
<td>11 (TmsBPU)</td>
<td></td>
<td><img src="image11" alt="Structure" /></td>
<td>0.38 ± 0.04</td>
<td>0.41 ± 0.05</td>
<td>nd</td>
<td>9.5</td>
</tr>
<tr>
<td>12 (TmUPS)</td>
<td></td>
<td><img src="image12" alt="Structure" /></td>
<td>Nd</td>
<td>7.16 ± 0.42</td>
<td>nd</td>
<td>79</td>
</tr>
<tr>
<td>13 (TmUPSB)</td>
<td></td>
<td><img src="image13" alt="Structure" /></td>
<td>Nd</td>
<td>0.98 ± 0.19</td>
<td>nd</td>
<td>15</td>
</tr>
<tr>
<td>14 (t-TUCB)</td>
<td></td>
<td><img src="image14" alt="Structure" /></td>
<td>0.19</td>
<td>0.77 ± 0.11</td>
<td>9 ± 1</td>
<td>5.2</td>
</tr>
<tr>
<td>15 (TPPU)</td>
<td></td>
<td><img src="image15" alt="Structure" /></td>
<td>0.57</td>
<td>0.73 ± 0.2</td>
<td>27 ± 2</td>
<td>34</td>
</tr>
</tbody>
</table>

Note: nd, not determined.

*a* IC50 was determined by the radiotracer assay using 50 μM t-DPPO as a radiometric substrate with 2.5 nM purified recombinant hsEH at 30 °C for 10 min. The result is the average of duplicates.

*b* IC50 was determined by the fluorescence assay using 5 μM CMNPC as a fluorescent substrate with 2.5 nM purified recombinant hsEH at 30 °C for 10 min. The result is the average of triplicates.
these are substrate based [5,11,21,44,45]. These substrate-based assays are attractive for mechanistic studies and allow one to prioritize the inhibitors with IC₅₀ values ranging from 0.4 to 100,000 nM. However, newer compounds have reached the limit of our fluorescent catalytic assay (IC₅₀ < 0.4 nM). Although other substrate-based assays, such as those using [³H] t-DPPO [14] (radiometric based) and 14,15-EET (LC–MS/MS assay) as a substrate, are able to distinguish among these potent compounds when [S] > [Kₘ], they are expensive and labor-intensive. The displacement assay is in simple equilibria among the inhibitor, ACPU, and sEH without the complication of the substrate catalysis in substrate-based assays. In addition, the displacement assay allows one to directly obtain the real affinity between inhibitors and enzyme and to measure the compounds with Kᵢ down to 0.01 nM, which current substrate-based assays are unable to do.

To compare the displacement assay with different substrate-based assays, we tested the same series of compounds in the displacement assay against different substrate-based assays (Table 1). The IC₅₀ values we obtained from different substrate-based assays show some discrepancies, and this observation is consistent with the study of Tsai and coworkers [46]. The plot of Kᵢ obtained from the displacement assay against IC₅₀ values from different assays shows that most of outlying points on the correction plot of IC₅₀ values are compounds with IC₅₀ values less than 5 nM with the fluorescent cyan(6-methoxy-naphthalen-2-yl)methyl trans-[(3-phenyloxynaran-2-yl)methyl]carbonate (CMNPC) assay (Fig. S7). This suggests that many newly developed inhibitors have reached the limit of the fluorescent substrate assay to distinguish among them. Based on the Cheng–Prusoff equation, increasing substrate concentrations can increase the experimental IC₅₀ values of inhibitors. Because the radiometric assay and the LC–MS/MS assay use approximately 10 times higher concentrations of substrate than the Kₘ value of the respective substrate (Table S2), these assays can distinguish among very potent compounds better than the fluorescent CMNPC assay. Based on the Spearman’s rank correlation coefficient analysis between the displacement assay and the other substrate-based assays (Table 2), the FRET displacement assay reported here correlated well with t-DPPO and 14,15-EET substrate-based assays and less well with the CMNPC fluorescent substrate assay. Thus, the CMNPC fluorescent substrate assay is a good way to rank compounds quickly, but reliability decreases as IC₅₀ values approach 5 nM, whereas the displacement assay provides a new way to distinguish among very potent sEH inhibitors.

Tsi and coworkers hypothesized that the discrepancy between fluorescent assay and radiometric assay was due to part to structural differences among inhibitors [46]. Here, compounds with different linkers, cyclohexyl-ether and piperidinyl, and substitutes on the linker and sEH without the complication of the substrate catalysis in substrate-based assays. In addition, the displacement assay allows one to directly obtain the real affinity between inhibitors and enzyme and to measure the compounds with Kᵢ down to 0.01 nM, which current substrate-based assays are unable to do.

To compare the displacement assay with different substrate-based assays, we tested the same series of compounds in the displacement assay against different substrate-based assays (Table 1). The IC₅₀ values we obtained from different substrate-based assays show some discrepancies, and this observation is consistent with the study of Tsai and coworkers [46]. The plot of Kᵢ obtained from the displacement assay against IC₅₀ values from different assays shows that most of outlying points on the correction plot of IC₅₀ values are compounds with IC₅₀ values less than 5 nM with the fluorescent cyan(6-methoxy-naphthalen-2-yl)methyl trans-[(3-phenyloxynaran-2-yl)methyl]carbonate (CMNPC) assay (Fig. S7). This suggests that many newly developed inhibitors have reached the limit of the fluorescent substrate assay to distinguish among them. Based on the Cheng–Prusoff equation, increasing substrate concentrations can increase the experimental IC₅₀ values of inhibitors. Because the radiometric assay and the LC–MS/MS assay use approximately 10 times higher concentrations of substrate than the Kₘ value of the respective substrate (Table S2), these assays can distinguish among very potent compounds better than the fluorescent CMNPC assay. Based on the Spearman’s rank correlation coefficient analysis between the displacement assay and the other substrate-based assays (Table 2), the FRET displacement assay reported here correlated well with t-DPPO and 14,15-EET substrate-based assays and less well with the CMNPC fluorescent substrate assay. Thus, the CMNPC fluorescent substrate assay is a good way to rank compounds quickly, but reliability decreases as IC₅₀ values approach 5 nM, whereas the displacement assay provides a new way to distinguish among very potent sEH inhibitors.

Table 2: Spearman’s rank correlation coefficient across different sEH catalytic assays.

<table>
<thead>
<tr>
<th>Reporter</th>
<th>CMNPC</th>
<th>[³H]t-DPPO</th>
<th>14,15-EET</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACPU</td>
<td>.75</td>
<td>.88</td>
<td>.92</td>
</tr>
<tr>
<td>CMNPC</td>
<td>–</td>
<td>.86</td>
<td>.65</td>
</tr>
<tr>
<td>[³H]t-DPPO</td>
<td>–</td>
<td>–</td>
<td>.90</td>
</tr>
</tbody>
</table>

**Development of FRET assay to measure relative k<sub>off</sub> of sEH inhibitors**

In addition to the Kᵢ, which reflects the strength of drug–target interaction, the drug–target residence time of slow tight binding inhibitors has become another vital parameter for choosing leads among compounds for optimization in medicinal chemistry and testing in vivo [22,47]. Because the drug is effective only when the inhibitor is bound to the target protein or enzyme, determining the residence time, which describes the period of time when the drug is bound to enzyme, is important. Residence time should be an improved metric for predicting the in vivo potency because in vivo models are an open system compared with in vitro enzyme assays in which the Kᵢ is measured in a closed system. In addition, Kᵢ is the ratio of k<sub>on</sub> to k<sub>off</sub>. Because k<sub>off</sub> of very potent compounds is limited by the diffusion of the molecules and k<sub>off</sub> is solely the dissociation of the inhibitor from the enzyme, which is usually slow for potent inhibitors, a method to measure k<sub>off</sub> is invaluable. Here, inspired by the application note of Invitrogen’s LanthaScreen Eu kinase binding assay kit, the FRET assay described above was further developed to measure the relative k<sub>off</sub> of sEH inhibitors. When the enzyme–inhibitor complexes are diluted, the inhibitor is dissociated from the enzyme and the free enzyme is trapped by a large excess of ACPU quickly and FRET occurs (see Scheme S1 in Supplementary material). By monitoring the FRET signal (excitation at 280 nm and emission at 450 nm) over time, we can determine k<sub>off</sub> of the sEH inhibitor through fitting to a first-order exponential growth curve (Fig. 4). The fastest inhibitor takes approximately 5000 s (~80 min) to be completely dissociated from the enzyme. Therefore, only the data obtained during the first 5000 s was used for determining k<sub>off</sub> (Table 3). In addition, a relative short time of analysis reduces the risk of secondary reactions, such as the rebinding of inhibitor to sEH and the degradation of enzyme–inhibitor complex, that could affect the analysis.

To determine whether 40 times dilution of the enzyme–inhibitor complex with a 100-fold excess of ACPU can bind the free enzyme quick enough to prevent the inhibitor from displacing...
the enzyme–ACPU during the first 5000 s, we conducted an experiment by varying the amount of ACPU (50- and 200-fold excesses) to dilute enzyme–inhibitor complex (Table 4). If the condition we used is not high enough to prevent the inhibitor from displacing the sEH–ACPU complex, the observed $k_{\text{off}}$ will be greatly affected by dilution of the complex with different amounts of ACPU. Although we observed a slight increase of the $k_{\text{off}}$ value when we increased the amount of ACPU used for dilution of the enzyme–inhibitor complex from 50-fold to 100-fold excess of ACPU, we did not observe much change of the $k_{\text{off}}$ value when we further increased the amount of ACPU from 100-fold excess to 200-fold excess. The results indicated that 100-fold excess of ACPU is enough to prevent the displacement of ACPU from enzyme–ACPU complex by inhibitor.

The observed $k_{\text{off}}$ for hsEH showed that it is well correlated with the size of the inhibitor but not the $K_i$ of the inhibitor. As the size of the inhibitor increased, the observed $k_{\text{off}}$ decreased (slower). We found that the adamantyl group on the left side of the urea gives the slowest $k_{\text{off}}$, followed by 4-trifluoromethoxyphenyl and trifluoromethyl (Table 3, from second to fourth entries and sixth to seventh entries). In addition, we found that sulfonamide on the piperidine linker leads to release from the hsEH much faster than the amide (Table 3, second and fifth entries).

**Table 3**

$k_{\text{off}}$ measurements of different sEH inhibitors from sEH from different species.

<table>
<thead>
<tr>
<th>Inhibitor number (and name)</th>
<th>sEH inhibitor structure</th>
<th>Human sEH</th>
<th>Mouse sEH</th>
<th>Rat sEH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (AUDA)</td>
<td><img src="image" alt="AUDA structure" /></td>
<td>0.9/13</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2 (APAU)</td>
<td><img src="image" alt="APAU structure" /></td>
<td>1.9/6</td>
<td>0.37/31</td>
<td>0.29/40</td>
</tr>
<tr>
<td>8 (TmPAU)</td>
<td><img src="image" alt="TmPAU structure" /></td>
<td>3.0/4</td>
<td>1.7/7</td>
<td>0.93/12</td>
</tr>
<tr>
<td>16 (TPAU)</td>
<td><img src="image" alt="TPAU structure" /></td>
<td>2.6/4</td>
<td>1.9/6</td>
<td>0.97/12</td>
</tr>
<tr>
<td>12 (TmUPS)</td>
<td><img src="image" alt="TmUPS structure" /></td>
<td>2.3/5</td>
<td>1.2/10</td>
<td>0.5/23</td>
</tr>
<tr>
<td>4 (t-AUCB)</td>
<td><img src="image" alt="t-AUCB structure" /></td>
<td>0.38/30</td>
<td>0.44/26</td>
<td>0.23/50</td>
</tr>
<tr>
<td>14 (t-TUCB)</td>
<td><img src="image" alt="t-TUCB structure" /></td>
<td>0.72/16</td>
<td>0.49/24</td>
<td>0.98/12</td>
</tr>
<tr>
<td>15 (TPPU)</td>
<td><img src="image" alt="TPPU structure" /></td>
<td>1.1/11</td>
<td>0.58/20</td>
<td>0.91/13</td>
</tr>
<tr>
<td>9 (TPCU)</td>
<td><img src="image" alt="TPCU structure" /></td>
<td>0.66/18</td>
<td>0.49/24</td>
<td>nd</td>
</tr>
<tr>
<td>13 (TmUPSB)</td>
<td><img src="image" alt="TmUPSB structure" /></td>
<td>1.0/12</td>
<td>0.42/28</td>
<td>0.36/32</td>
</tr>
</tbody>
</table>

Note: The preincubated enzyme–inhibitor (8 μM) was diluted 40 times with 100 equivalents of ACPU (20 μM, 100 mM sodium phosphate, pH 7.4) at 25 °C. nd, not determined.

**Determination of $K_i$ and $k_{\text{off}}$ of sEH inhibitors from other species sEH with ACPU**

Based on the sequence analysis, rodent and murine sEH showed greater than 76% sequence identity to hsEH. Therefore, ACPU should be able to measure $K_i$ of sEH inhibitors on sEH from other species. Although we were able to determine $K_i$ of the reporter on both murine sEH ($K_d = 0.1$ nM) and rat sEH ($K_d = 0.17$ nM), we were unable to completely displace the bound ACPU from the rat sEH even with an excess of very potent sEH inhibitor (t-TUCB) within a reasonable period of time (3 h). Therefore, we could obtain only the $K_i$ values of sEH inhibitors against murine sEH (compound: 2, 1.86 nM; compound 14, 6.22 nM; and compound 13, 1.08 nM). Based on these results, mouse sEH (msEH) binds sEH inhibitors with an adamantyl substituent much tighter than hsEH (compound 2, hsEH = 21.7 nM, msEH = 1.86 nM).
In addition, using ACPU, $k_{\text{off}}$ values for sEH inhibitors against both murine and rat sEH were obtained (Table 3). Compounds containing adamantyl group had a much slower $k_{\text{off}}$ for mouse sEH and rat sEH as compared with human sEH. These results suggest that there may be difficulty in translation of the efficacy of sEH inhibitors obtained from in vivo studies to human. This enzyme has different $K_i$ and $k_{\text{off}}$ values as compared with those of rat and mouse.

**Table 4**

$k_{\text{off}}$ measured from hSEH with different equivalents of ACPU.

<table>
<thead>
<tr>
<th>Inhibitor number (and name)</th>
<th>50-Equivalent excess of ACPU</th>
<th>100-Equivalent excess of ACPU</th>
<th>200-Equivalent excess of ACPU</th>
</tr>
</thead>
<tbody>
<tr>
<td>observed $k_{\text{off}}$ ($10^{-3}$ s$^{-1}$)</td>
<td>3.0</td>
<td>0.38</td>
<td>0.40</td>
</tr>
<tr>
<td>4 [r-AUCB]</td>
<td>0.54</td>
<td>0.58</td>
<td>0.54</td>
</tr>
<tr>
<td>11 (TmsBPU)</td>
<td>1.1</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>1 (APAU)</td>
<td>2.0</td>
<td>2.2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Note: The preincubated enzyme–inhibitor (8 μM) was diluted 40 times with different equivalents of ACPU in PB buffer (100 mM sodium phosphate, pH 7.4) at 25 °C.*

**Conclusion**

Here, we have reported a new fluorescent reporter, ACPU, that can be used to determine the accurate $K_i$ of sEH inhibitors by competitive displacement assay down to sub-nanomolar or picomolar levels using a 96-well plate-based assay or a cuvette-based assay, respectively. In addition, ACPU was further used for $k_{\text{off}}$ determination of sEH inhibitors. $k_{\text{off}}$ has become an important parameter to predict in vivo potency. This assay is the first reported fluorescence-based assay to measure the relative $k_{\text{off}}$ for sEH inhibitors and has been adapted to a 96-well plate format that can provide an alternative way to screen and measure the potency of sEH inhibitors.

During the assay development, several parameters were optimized for effective $K_i$ or $k_{\text{off}}$ measurement for potent inhibitors. $K_i$ of the reporting ligand must be low, and the quantum yield for the reporting ligand must be high, so that a low concentration of the targeted enzyme can be used. In addition, $k_{\text{on}}$ of the reporting ligand must be fast enough to yield a practical $k_{\text{on}}$ measurement. However, the $k_{\text{off}}$ of the reporting ligand should not be too slow; otherwise, it would be impractical for $K_i$ determination.

Whereas the previous substrate-based assays either are unable to measure the $K_i$ for sEH inhibitors or are labor-intensive, the current competitive displacement assay is easier and cheaper to use. In addition, we showed that the current assay could be adapted to a 96-well plate format that can facilitate the screening of newly synthesized sEH inhibitors, which usually are too potent for CMNPC fluorescent assay to distinguish among them.

It is suspected, albeit not proven, that fatty acid epoxides from ω-3 and ω-6 series are endogenous substrates [18]. These substrates will compete with sEH inhibitors in vivo as a function of concentration and properties of inhibitor, but they also will compete as a function of concentration and affinity of endogenous substrates. A good correlation between $K_i$ values obtained from FRET-based displacement assay and $IC_{50}$ values determined by LC–MS/MS using 14,15-EET as a substrate suggested that the FRET displacement assay could predict the in vivo potency for the inhibitors.

Based on these reasons, the FRET-based displacement assay reported here provides an alternative and possibly improved way to predict the in vivo potency of sEH inhibitors.

**Acknowledgments**

This work was partially funded by National Institute of Environmental Health Sciences (NIEHS) grant ES002710, NIEHS Superfund Research Program grant P42 ES04699, and National Institutes of Health (NIH) Counter Act Program U54 NS079202-01. B.D.H. is a George and Judy Marcus Senior Fellow of the American Asthma Foundation.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.ab.2012.11.015](http://dx.doi.org/10.1016/j.ab.2012.11.015).

**References**


