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

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ABSTRACT

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_{off}, 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-2*H*-chromen-4-yl)acetyl) piperidin-4-yl)urea (ACPU), was designed and synthesized. With ACPU, we have developed a Förster 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_{off} values of the inhibitors. This assay provides additional data to evaluate the potency of sEH inhibitors.

In mammals, the soluble epoxide hydrolase (sEH)[1] (EC 3.3.2.10) metabolizes important signaling epoxy fatty acids to the corresponding 1,2-diols [1]. These epoxy fatty acids, particularly epoxyeicosatrienoic acids (EETs) from arachidonic acid and epoxides from omega-3 fatty acids, have been demonstrated to maintain blood pressure in several animal models, to resolve inflammatory disorders and reduce pain, and generally to maintain homeostasis [2–9]. Therefore, stabilization of EETs through inhibition of sEH could be beneficial to human health. Over the past decade, significant progress has been made toward the clinical development of sEH inhibitors [10].

Catalytic assays for sEH have been instrumental in obtaining improved sEH inhibitors. Over the years, numerous substrate-based assays have been developed and used [11–20]. Existing sEH assays are able to distinguish among inhibitors of varying potency down to low nanomolar. However, as the inhibitor concentration approaches the enzyme concentration, the assays cannot distinguish among the most potent inhibitors. Several laboratories have reached this sensitivity limit with compounds they have made [5,10,21], so an assay capable of distinguishing among highly potent inhibitors would be attractive.

Catalytic assays for enzymes and their inhibitors are attractive for many mechanistic reasons; however, binding assays lend themselves to high-throughput formats easier than enzymatic assays. In general, the different sEH catalytic assays rank compounds in similar order, but there are notable exceptions. A binding assay with the enzyme in which catalysis plays no role could simplify data interpretation. Several binding assays have been described for sEH [13,16]. In 2009, Eldrup and coworkers reported the use of a tetramethyl rhodamine-labeled probe to measure human or rat sEH inhibition [13]. However, such an assay requires spectrometers that can measure fluorescence polarization in order to distinguish the bound probe from background. In a similar approach, urea-based inhibitors containing aryl groups are able to quench sEH intrinsic tryptophan fluorescence. However, to observe fluorescent quenching through binding to inhibitors, a relatively high

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[1] *Abbreviations used:* sEH, soluble epoxide hydrolase; EET, epoxyeicosatrienoic acid; IC_{50}, half-maximal inhibitory concentration; FRET, Förster resonance energy transfer; 14,15-EET, 14,15-epoxyeicosatrienoic acid; PTU, 1-(piperidine-4-yl)-3-(4-(trifluoromethyl)phenyl)urea; DMSO, dimethyl sulfoxide; ACPU, 1-(adamantan-1-yl)-3-(1-(2-(7-hydroxy-2-oxo-2*H*-chromen-4-yl)acetyl)piperidin-4-yl)urea; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, 4-dimethylaminopyridine; rt, room temperature; DMF, dimethylformamide; hsEH, human sEH; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PB buffer, sodium phosphate buffer; *t*-DPPO, *trans*-diphenyl-propene oxide; CMNPC, cyano(6-methoxy-naphthalen-2-yl)methyl *trans*-[(3-phenyloxyran-2-yl)methyl] carbonate; msEH, mouse sEH.

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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_{50}) values. Because the binding probes used in the above assay do not bind the sEH tightly (IC_{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-Epoxyeicosatrienoic 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-(trifluoromethyl) phenyl)ureido) piperidine-1-carboxylate, 1-(adamantan-1-yl)-3-(piperidin-4-yl)urea, 1-(piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl) urea (PTU), and 1-((1*r*,4*r*)-4-hydroxycyclohexyl)-3-(4-(trifluoromethoxy)phenyl)urea have been reported elsewhere [5,21,23,24]. All reactions for this study were carried out in a dry nitrogen atmosphere unless otherwise specified. Reactions were monitored by thin-layer chromatography on Merck F_{254} silica gel 60 aluminum sheets, and spots were either visible under light or ultraviolet light (254 mm) or stained with an oxidizing solution ($KMnO_4$ stain). Column chromatography was performed with silica gel.

^{1}H NMR spectra were recorded on a Varian QE-300 spectrometer with deuterated chloroform ($CDCl_3$, δ = 7.24 ppm) or deuterated dimethyl sulfoxide (DMSO-d_6) containing TMS as an internal standard. ^{13}C NMR spectra were recorded on a Varian QE-300 spectrometer at 75 MHz. ^{19}F NMR spectra were recorded on a Varian QE-300 spectrometer at 282.4 MHz.

Synthesis of 1-(adamantan-1-yl)-3-(1-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetyl)piperidin-4-yl)urea (ACPU)

1-(Adamantan-1-yl)-3-(piperidin-4-yl)urea (50 mg, 180 μmol) [23], 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 34.4 mg, 180 μmol), 4-dimethylaminopyridine (DMAP, 21.8 mg, 180 μmol), and 7-hydroxycoumarin-4-acetic acid (39.6 mg, 180 μmol) were dissolved in CH_2Cl_2 (50 ml). The reaction vessel was wrapped with aluminum foil, and the reaction mixture was stirred for 12 h at room temperature (rt). The reaction was quenched by the addition of aqueous HCl solution (0.1 M), and the organic layer was collected. The aqueous layer was extracted by CH_2Cl_2 five times, and the combined organic layers were dried over anhydrous $MgSO_4$. The crude product was concentrated and further purified by flash chromatography. The final product was eluted with 2% MeOH in EtOAc, resulting in pale yellow solid (56 mg, 116 μmol, 65% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.57 (br, 1H), 7.48 (d, *J* = 9 Hz, 1H), 6.77 (dd, *J* = 9 and 1 Hz, 1H), 6.71 (d, *J* = 1 Hz), 6.08 (s, 1H), 5.71 (d, *J* = 7.5 Hz, 1H), 5.42 (s, 1H), 4.08 (d, *J* = 14 Hz, 1H), 3.94 (s, 2H), 3.82 (d, *J* = 14 Hz, 1H), 3.55 (m, 1H), 3.20 (t, *J* = 11 Hz, 1H), 2.84 (t, *J* = 11 Hz, 1H), 1.99 (s, 3H), 1.85 (s, 6H), 1.76 (m, 2H), 1.59 (s, 6H), 1.11–1.30 (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). ^{1}H NMR (DMSO-d_6, 300 MHz): 8.77 (d, *J* = 8.4 Hz, 1H), 7.57 (s, 4H), 6.37 (s, 1H), 4.22 (m, 1H), 3.88 (d, *J* = 13.2 Hz, 1H), 3.71 (m, 1H), 3.17 (t, *J* = 12.8 Hz, 1H), 2.84 (m, *2*H), 1.85 (m, 2H), 1.54 (m, 1H), 1.29 (m, 3H), 0.97 (s, 3H), 0.81 (d, *J* = 6 Hz, 3H).

*Synthesis of 1-(1-(methanesulfonyl)piperidin-4-yl)-3-(4-(trifluoromethyl)phenyl)urea (**12**)</H2>*

PTU (70 mg, 0.244 mmol) and Et_3N (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). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.82 (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, *2*H).

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

Butylsulfonyl 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 (65 mg, 0.160 mmol, 66% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): ∂ 8.78 (s, 1H), 7.57 (s, 4H), 6.38 (d, *J* = 8 Hz, 1H), 3.82 (d, *J* = Hz, 1H), 3.62 (m, 1H), 3.50 (d, *J* = 12 Hz, 2H), 3.00 (m, 4H), 1.89 (d, *J* = 11 Hz, 2H), 1.66 (m, 2H), 1.40 (m, 4H), 0.90 (t, *J* = 8 Hz, 3H).

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_{50} determination for hsEH inhibitors

IC_{50} values of human sEH (hsEH) inhibitors were determined by three different assays (radiometric, fluorescent, and liquid chroma-