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Drug-Target Residence Time Affects *in Vivo* Target Occupancy through Multiple Pathways

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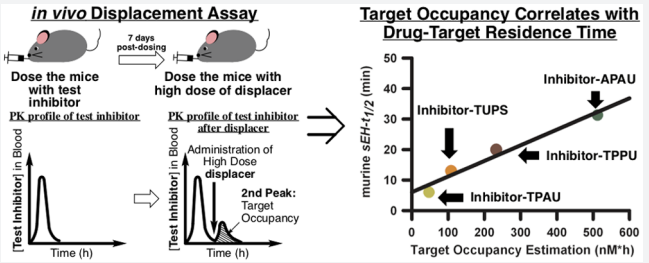
Supporting Information

ABSTRACT: The drug discovery and development process is greatly hampered by difficulties in translating *in vitro* potency to *in vivo* efficacy. Recent studies suggest that the long-neglected drug-target residence time parameter complements classical drug affinity parameters (K_I, K_d, IC_{50}, or EC_{50}) and is a better predictor of *in vivo* efficacy. Compounds with a long drug-target residence time are often more efficacious *in vivo*. The impact, however, of the drug-target residence time on *in vivo* efficacy remains controversial due to difficulties in experimentally determining the *in vivo* target occupancy during drug treatment. To tackle this problem, an *in vivo* displacement assay was developed using soluble epoxide hydrolase as a biological model. In this report, we experimentally demonstrated that drug-target residence time affects the duration of *in vivo* drug-target binding. In addition, the drug-target residence time plays an important role in modulating the rate of drug metabolism which also affects the efficacy of the drug.



INTRODUCTION

Our ability to predict accurately which small molecules will be efficacious in the clinic remains limited even after decades of research. Several studies showed that lack of efficacy remains a leading cause of drug failure in Phase II and Phase III clinical trials.[1−3] Although multiple factors often contribute to this outcome, a poor translation of *in vitro* potency as determined with pure enzymes or cell assays to *in vivo* efficacy in patients is proposed to be at the heart of this problem.[4−6] This discrepancy between *in vitro* potency and *in vivo* efficacy, particularly for small molecules, is partly because the standard *in vitro* potency parameters (K_d, K_i, IC_{50}, and EC_{50}) are measured in a closed system which is very different from the *in vivo* environment where a drug is exposed to an open system. Here, a drug is constantly interacting with different proteins, refractory pools, metabolism, and excretion in addition to its own target. Therefore, a complementary *in vitro* parameter that accounts for the kinetic aspect in an open system helps us to better translate the *in vitro* potency of the drug to *in vivo* efficacy.

Recently, as technologies to measure the kinetic potency of an inhibitor or a ligand, k_{off}, become more easily accessible,[7−10] more studies have suggested that drug-target residence time (t_R), which is the reciprocal of the dissociation rate constant of a drug-target complex, is a better in vitro parameter to predict in vivo efficacy of a drug. This is because a drug is only active when it is bound to its target; therefore, a long t_R prolongs the drug-target interactions and extends the biological effects of the drug.[11−18] Although numerous studies have demonstrated that a drug with a long t_R has improved and extended its *in vivo* activity in animal models, which can be translated to its efficacy, a detailed molecular mechanism on the effect of t_R on *in vivo* pharmacological activity of a drug remains unclear. For example, because K_d or K_i is a ratio of k_{off} over k_{on}, an improvement of the k_{off} of the drug, which dictates the t_R of the drug, often leads to an improvement of the binding affinity (K_d and K_i) of the drug. In addition, there is no current method that could estimate the duration of drug-target binding *in vivo*. Thus, it is difficult to decipher whether the enhanced *in vivo* activity of a drug is due to an improvement in the binding affinity constant, the t_R of the drug, or a combination of both. Therefore, how the t_R affects drug efficacy remains under investigation.

In this report, we studied the effect of t_R on drug-target interaction*in vivo* by developing an *in vivo* displacement assay to monitor the duration of drug-target binding *in vivo*. To develop an assay correlated with our *in vitro* t_R assay, a well-defined biological system was needed. The soluble epoxide hydrolase (sEH) was chosen as our model system because sEH, a tissue localized enzyme, has been well-studied for decades and has an important regulatory role in inflammation,

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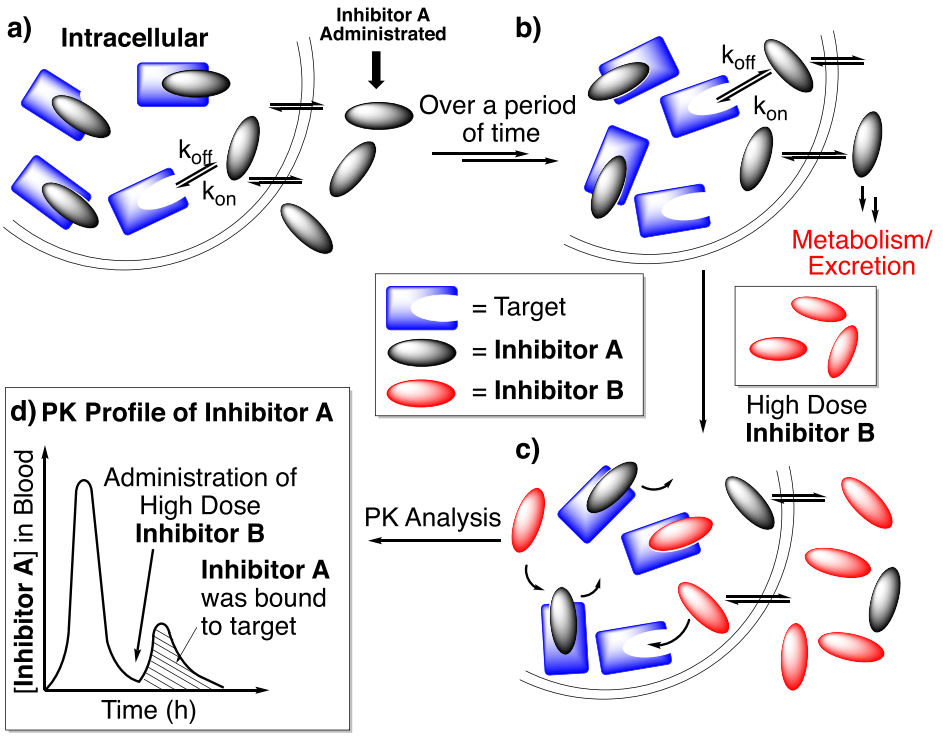

Figure 1. Schematic diagram of the *in vivo* displacement assay. (a) Step 1, inhibitor A binds to the target enzyme after administration. (b) Step 2, the administrated inhibitor A *in vivo* is metabolized and/or excreted over time. (c) Step 3, high dose of displacement inhibitor B is administrated. The bound inhibitor A is competed and displaced by high concentration of inhibitor B. Inhibitor A is released to the blood and can be monitored by LC/MSMS. (d) Step 4, the expected PK profile of the *in vivo* displacement assay. The first peak in the PK profile corresponds to the blood concentration of inhibitor A after inhibitor A administration. The second peak of the PK diagram is hypothesized as the bound inhibitor A displaced by a high dose of inhibitor B. The area-under-the-curve (AUC) of the second peak reflects the amount of soluble epoxide hydrolase bound inhibitor A *in vivo*.

blood pressure, pain perception, and angiogenesis, which makes it a good drug target.[19−22] Over decades of research, several tools developed for sEH research have proved invaluable for studying drug-target residence time of sEH inhibitors, including (1) a large library of sEH inhibitors (≥3000 compounds), which are highly diversified in potency, physical properties, and pharmacokinetic (PK) parameters; (2) an established analytical method with a low limit of quantification (LOQ ≤ 0.5 nM) that allows for a quantification of drug concentration in blood and tissues; (3) highly pure sEH recombinant enzymes from different species; (4) high-throughput assays for measuring both the K_i and t_R of the inhibitors of sEH that have recently been established;[23] and (5) a sEH knockout mouse that can be used as a negative control to both validate the results and to decipher the effect of target occupancy on the PK profile of the inhibitors of sEH.

Here, we report our results using an *in vivo* displacement assay which allows us to estimate the amount of target-bound sEH inhibitor *in vivo*. With this assay, we demonstrated that the t_R of the inhibitor directly influences the duration of inhibitor-target protein interaction *in vivo*. In addition, this PK study revealed that the t_R of the inhibitor affects its PK profile by protecting it from metabolism. These results suggest that t_R affects the *in vivo* efficacy of the drug through multiple pathways.

RESULTS

Design and Development of an *in Vivo* Displacement Assay to Estimate the Amount of Bound sEH Inhibitor *in Vivo*As a Function of Time. To determine whether t_R affects the duration of drug-target occupancy *in vivo*, it must be feasible to monitor the amount of drug bound to the target protein at a specific time point. Therefore, we developed an *in vivo* displacement assay that could estimate the amount of bound drug *in vivo* as shown in Figure 1. Briefly, the compound of interest is administrated to the animals to reach a target *in vivo* level (C_{max}) that is at least 100 times above the K_i of the compound. This is referred to as the loading compound and loading dose. This is to ensure near maximum binding of the compound to the target protein *in vivo*. After a long postdosing period, when the compound of interest has been largely metabolized and/or excreted, a second compound, which is very potent and selective for the target enzyme, is administrated to the same animals at a high dose. This second compound is the displacement compound given at the displacement dose. We hypothesized that if the initial loading compound of interest (inhibitor A, Figure 1b) remains bound to the target protein *in vivo* after a long postdosing period, the bound inhibitor will be displaced by the second high-affinity compound (inhibitor B, Figure 1c), and the displaced inhibitor A will be returned into the circulation. Thus, the blood level of the inhibitor A will increase (second displacement peak) after the administration of a high dose of a second high-affinity compound (Figure 1c,d). Because of the sensitivity of our analytical method (LOQ ≥ 0.49 nM), the compound level in the blood is easily monitored by LC/MS-MS.

***In Vivo* Displacement Assay Can Estimate the Amount of sEH Inhibitor Specifically Bound to sEH in Vivo.** For assay development, the sEH inhibitor, 3-(4-

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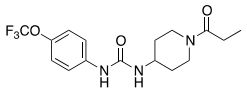
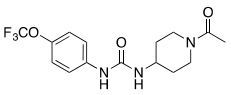
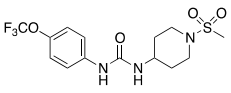
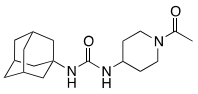
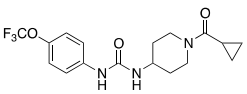
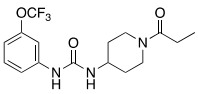
Table 1. In Vitro and in Vivo Parameters of sEH Inhibitors Used in the Mouse Study

Structure	[a] LogP	[a] Ki (nM)	[a] sEH-$t_{1/2}$ (min)	[b]PPB (%)	Plasma to Blood Ratio[c]	[d,e] Dose (mg/kg)	[d,e] AUC (nM*h)	[d,e] Cmax (nM)	[d,e] PK-$t_{1/2}$ (h)	[d,e] T_{max} (h)
 TPPU	3.23	2.50± 0.38	19.8± 0.2	79±1	0.48± 0.06	0.3	8813	456	13.0	1.0
 TPAU	2.99	4.33± 0.13	6.0	80±3	0.39± 0.01	1	13783	830	7.8	4.7
 TUPS	2.99	2.09± 0.32	10.0± 0.4	84±1	0.68± 0.07	0.3	11199	219	19.1	4.5
 APAU	2.10	1.88± 0.02	31.3± 1.9	50±2	1±0.1	20	24491	6444	1.4	1.1
 TCPU	3.28	0.92± 0.09	23.8± 2.7	90±1	N.D.[f]	3	56254	4114	3.7	4.7
 mTPPU	3.10	≥10 μM*	N/A	N.D.[f]	N.D.[f]	3	1504	1019	0.4	0.9

[a]LogP, K_i, and $t_{1/2}$ were reported before, and K_i and $t_{1/2}$ were measured with murine sEH.[22] The results are the average of triplicates with ± SEM. [b]Plasma protein binding (%) was reported by Lee et al.[23] [c]The plasma-to-blood-ratio was an average of duplicates with ± SEM. [d]The mice (n = 3−6) were treated by subcutaneous injection with inhibitor at the desired dose (0.3−20 mg/kg dissolved in PEG400). [e]The pharmacokinetic parameters were calculated by Winonlin based on the best-fitted models. [f]N.D. stands for not determined. Abbreviation: elogP stands for experimental log P which was determined by the HPLC method in Lee et al.[24] Similar data for compounds used in rat study are provided in Table S2.

(trifluoromethoxy)-1-(propionlpiperidin-4-yl)-phenyl)urea (TPPU), was chosen as the compound of interest (loading compound or inhibitor A) because it is potent (K_i = 2.5 nM) with reasonable t_R (t_R = 28.6 min), and the sEH-TPPU dissociation half-life (sEH-$t_{1/2}$ = 19.8 min) has a relatively long PK elimination half-life (PK-$t_{1/2}$, 13.0 h) and is widely used as a tool compound in sEH research.[23,24] The long PK-$t_{1/2}$ ensures that the inability to detect TPPU being displaced from the target protein is not due to rapid metabolism (Table 1). For the ease of comparison between the *in vitro* kinetic and *in vivo* elimination half-life, we will focus our discussion regarding the data using sEH-$t_{1/2}$ which is ln 2 *t_R instead of t_R. In addition, TCPU was selected as displacement compound or inhibitor B to displace any bound TPPU in vivo because it is also very potent (K_i = 0.92 nM, sEH-$t_{1/2}$ = 23.8 min) with a reasonable PK exposure at 3 mg/kg (C_{max} = 4114 nM, area-under-the-curve (AUC) = 56 254 nM·h, Table 1). These properties ensure that the sEH-bound TPPU can be displaced by a high in vivo concentration of TCPU.[24,25] Therefore, one can ensure that there would be a high level of TCPU for a long period of time in vivo to displace all the sEH-bound TPPU in vivo (Table 1). Experiments testing the hypothesis that the sEH inhibitor loaded on the target enzyme can later be displaced by a second compound are presented in Figures 2, 3, and 4 and S1−S18. On the basis of the published PK profile of TPPU, a 0.3 mg/kg dose was used because it provided a high exposure of TPPU *in vivo* with C_{max} at 456 nM (~182-fold of K_i) and AUC at 8813 nM·h. In addition, this dose of TPPU has demonstrated pharmacological activity in several animal models.[26−28] As mentioned by Morisseau et al., in order to have significant effect on epoxide hydrolysis by sEH *in vivo*, at least 90% of sEH needs to be inhibited.[29] These results indicated that the majority of sEH is inhibited by TPPU at dose of 0.3 mg/kg. A PK profile of TPPU at 0.3 mg/kg showed (Figure 2a) that TPPU reached a sustained near zero level (~15 nM) in blood at 168h (Figure 2a, post dosing day 7) indicating that a majority of free TPPU had been metabolized or excreted from the body. We refer to this as the terminal phase. (Figure S1) Here we hypothesize that there is a significant amount of TPPU remaining *in vivo* and mostly

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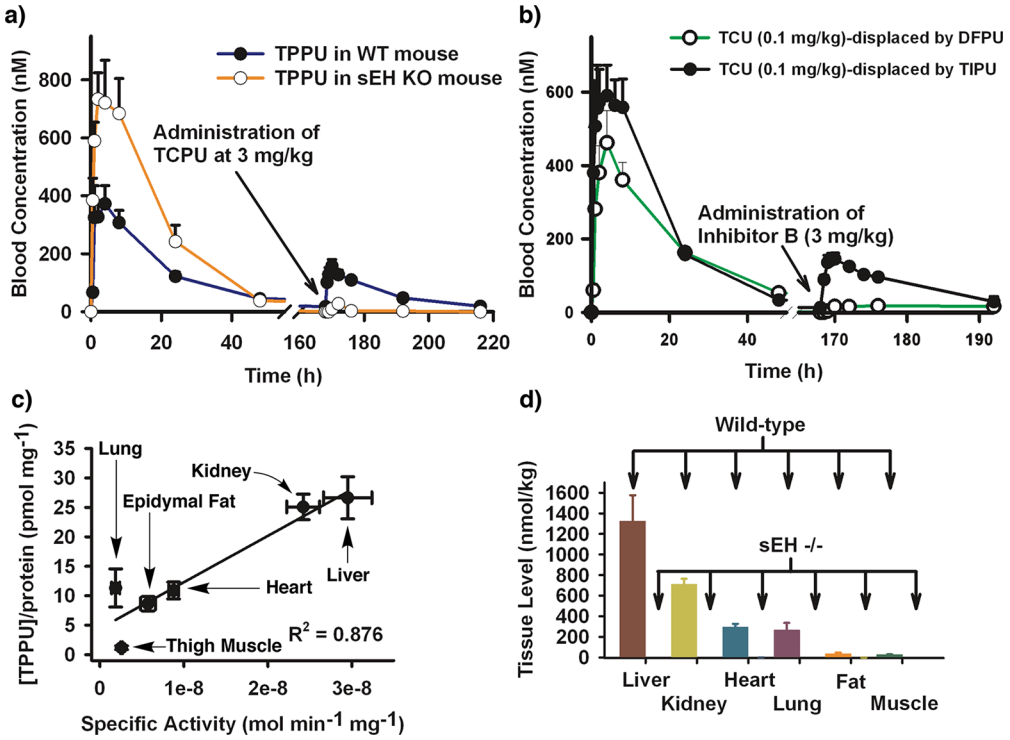

Figure 2. (a) The *in vivo* displacement assay between the WT mice (black line with solid circle, $n = 6$) and sEH KO mice (orange line with open circle, $n = 6$) of TPPU (0.3 mg/kg in PEG400, 100−110 μL based on the weight of the mouse, subcutaneous injection) indicates that the second displacement peak of TPPU in WT mice after administration of a high dose of the potent displacement sEH inhibitor (TCPU, 3 mg/kg in PEG400, 100−110 μL based on the weight of the mouse, subcutaneous injection) at 168 h, is the sEH-bound TPPU. For the structure of TPPU and TCPU, please refer to Table 1. (b) The result from the *in vivo* displacement assay in rat where the TCU (0.1 mg/kg in PEG400/olelate rich safflower oil/1:4, 1 mL, oral gavage) was displaced by a high dose of the weak inhibitor, DFPU (green line with open circle, $n = 4$, 3 mg/kg in PEG400/olelate rich safflower oil/1:4, 1 mL, oral gavage), or a high dose of potent inhibitor, TIPU (black line with solid circle, $n = 4$, 3 mg/kg, PEG400/olelate rich safflower oil/1:4, 1 mL, oral gavage), at 168 h indicates that the second displacement peak of TCU in rats is the sEH-bound TCU. For the structure of TCU, TIPU, and DFPU, please refer to Table S2. Panels (a, b) indicate that the *in vivo* displacement assay can be applied to other species. (c) TPPU level at postdosing day 7 correlated well with specific sEH activity in different tissues in WT mice. Please see Figure S15 for the relationship between the TPPU tissue level and sEH activity in different tissues in WT mice. R^2 was calculated based on the datum point close to the fitted line (black) except for thigh muscle. (d) The tissue level of TPPU at postdosing day 7 in different organs in WT mouse and sEH KO mouse ($n = 6$ per group). Unlike the WT mouse, there was no accumulation of TPPU in the sEH KO mouse. Please see Figure S16 for the tissue level of TPPU at postdosing day 7 in different organs in the Sprague−Dawley rat. The data are mean ± standard error of the mean (SEM).

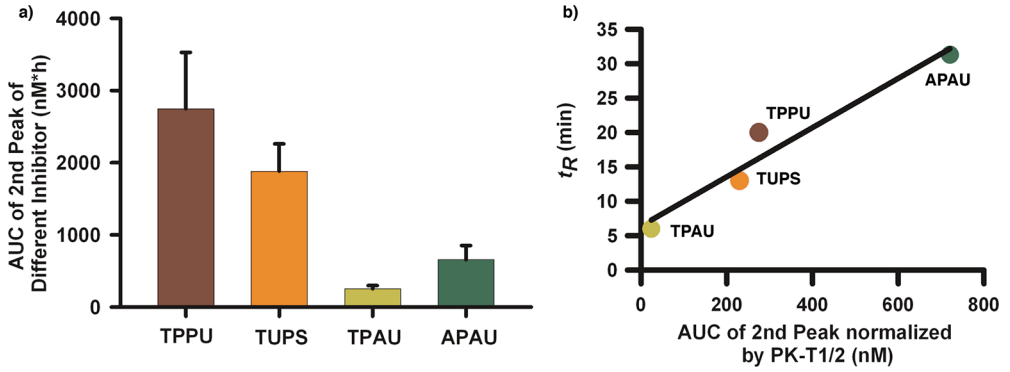

Figure 3. t_R of the inhibitors impact the AUC of the second displacement peak (referenced to the displaced sEH-bound inhibitor) at day 7. (a) The AUC of the second displacement peak (the tested inhibitor displaced by a high dose of TCPU) changed with the t_R of the tested inhibitor in the *in vivo*displacement assay. The AUC of second peak correlates well with inhibitors t_R except APAU which has a much shorter PK-$T_{1/2}$. The experimental protocol is detailed in Supporting Information. The data are mean ± SEM. (b) After normalization of the second peak AUC with PK-$T_{1/2}$ of the same inhibitor, the normalized AUC of the second peak correlates well ($R^2 = 0.962$) with the t_R of the inhibitors.

bound to the sEH. Therefore, it is not available for circulation. To estimate the amount of sEH-bound TPPU remaining in vivo, a high dose of displacement compound, TCPU was administrated to displace the sEH-bound TPPU of the sEH. Because the level of TPPU in the blood at postdosing day 7 was low, this treatment would enhance our chance to observe any changes of the blood level of TPPU resulting from the sEH-bound TPPU displaced by TCPU. In addition, because the *in vivo* level of TPPU is stable after postdosing day 7 (Figure 2a), any subtle changes of the time of the

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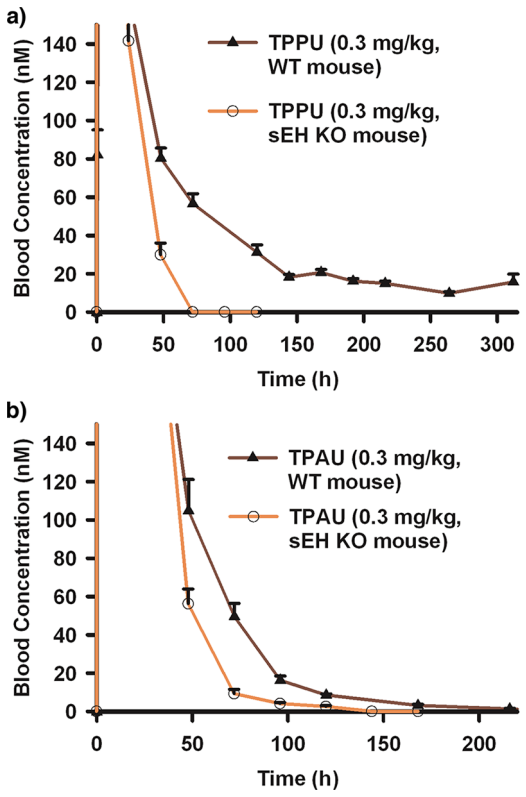

Figure 4. Drug-target residence time (t_R) modulates the pharmacokinetic profile of the inhibitors. The inhibitors with longer t_R bind to the sEH longer as compared to inhibitors with shorter t_R, therefore protecting the inhibitors from being metabolized and/or eliminated from the body. (a) TPPU, which has a long t_R (19.8 min), was administrated to both WT and sEH KO mice at the same dose (subcutaneous injection, 0.3 mg/kg). The PK profile of TPPU in WT mice (PK-T1/2 is 15 h) is very different from one in sEH KO mice (PK-T1/2 is 9 h) after 48 h. The TPPU is fully eliminated in sEH KO mouse within 72 h, while the elimination rate of TPPU is much slower in WT mice and the blood level of TPPU stayed above the K_i (2.5 nM) even at postdosing day 14. (b) TPAU, which has a short t_R (6.0 min), was administrated to both WT and sEH KO mice at the same dose (1 mg/kg). Unlike TPPU, the PK profiles of TPAU between WT (PK-T1/2 is 8.75 h) and sEH KO mice (PK-T1/2 is 8.35 h) is relatively similar to TPAU and is fully eliminated in WT mice within 168 h, while TPAU is fully eliminated in sEH KO mice within 144 h. These data suggest that t_R of the inhibitors affects its PK profiles, and inhibitors with a long t_R will have a slower PK elimination rate. Please refer to Figure S17 for a full PK profile of TPPU and TPAU in WT and sEH KO mice. The data are mean ± SEM.

administration of TCPU would not lead to a significant change of the second displacement peak in the PK profile.

As Figure 2a shows, the level of TPPU in the blood increased immediately (second displacement peak of TPPU) after a high dose of a displacement compound, TCPU (3 mg/kg), was administrated indicating that a substantial amount of TPPU remained bound to the sEH in vivo. We then analyzed the tissue level of TPPU in order to understand whether TPPU was stored in specific organs and if the level correlates well with the sEH activity in the specific tissue. As shown in Figures 2c and S2, the amount of the TPPU found in the specific tissues correlated very well with the sEH activity of the same tissue. This work was also repeated with TCU as a loading compound in rat (Figure S9). Therefore, we hypothesize that the increase in the level of the second displacement peak of TPPU followed by administration of a high dose of TCPU corresponds to the amount of TPPU that is bound to sEH.

To test the hypothesis that the second displacement peak of TPPU in the *in vivo* displacement assay discussed above is due to the sEH-bound TPPU, similar experiments were run with the following modifications: (1) where TCPU is replaced by 3-(3-(trifluoromethoxy)-1-(propionlpiperidin-4-yl)-phenyl)urea (mTPPU) (Figure S3) which is >10000 less potent than TCPU, and (2) WT mice are replaced with sEH knockout (KO) mice. As shown in Figures 2a and S3, our results showed that a much smaller second peak was observed in both experiments (Figures 2a and S3). In addition, in a similar experimental setting with sEH knockout mice, an increase in the dose of TPPU from 0.3 mg/kg to 3 mg/kg and an increase in the dose of TCPU from 3 mg/kg to 10 mg/kg did not further increase the area-under-the-curve (AUC) of the second displacement peak of TPPU (Figure S4). Furthermore, as shown in Figure 2d, unlike WT mice, at postdosing day 7, no TPPU is accumulated in sEH KO mice tissues. All results implied that the second displacement peak of TPPU observed in the experiment with WT mouse after administration of TCPU (Figure 2a) is due largely to the sEH-bound TPPU in vivo.

To determine if a near optimum dose of TCPU was used in the *in vivo* displacement assay, a dose response study was conducted. Our results showed that the AUC of the second displacement peak reached plateau at 3 mg/kg, which indicates that majority of the sEH-bound TPPU was displaced and 3 mg/kg of TCPU is a near optimum dose of the displacement assay (Figures S5−S8).

To test if this assay can also be applied to other model species, the same assay was additionally performed in rats with inhibitors optimized for rat enzyme and PK. As shown in Figure 2b, we were also able to displace specifically the bound sEH inhibitor (TCU) with a high dose of a second inhibitor (TIPU) in rats. When a much less potent DFPU (~16 666-fold less potent, Table S1) or vehicle alone was used instead of TIPU, the TCU could not be displaced (Figures 2b, S9 and S10). These results indicate that the second displacement peak corresponds to the amount of sEH-bound TCU *in vivo*. Therefore, the *in vivo* displacement assay developed here is likely broadly transferable to other species and other compound pairs.

Drug-Target Residence Time Affects the Duration of the *in Vivo* Drug−Target Interactions. This newly developed *in vivo* assay allows one to monitor the amount of bound inhibitor at a specific time after the dosing of the drug. Such information can be extrapolated to the time-dependent drug's pharmacological activity *in vivo* because the inhibitor is only active when it is bound to the target. To study the correlation of *in vitro* t_R on the duration of drug-target interaction *in vivo*, a subset of metabolically stable sEH inhibitors with similar K_i and physical properties but different sEH-$t_{1/2}$ (which implies the same as t_R) values was identified from our sEH inhibitor library (Table 1). This set of sEH inhibitors allows one to study specifically the effect of t_R on the duration of *in vivo* drug-target interactions with minimal interference from other factors. This approach is important for our study because, in most cases, modulating t_R will affect the K_i of the inhibitor because K_i is a ratio of k_{off} over k_{on}. In addition, similar to previous experiments, we used a dose of inhibitor where C_{max} is at least 100 times higher than the K_I,

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and the AUC (exposure) is ≥8000 nM·h (Table 1) to ensure the majority of the sEH is bound to the inhibitor.

As shown in Figure 3, our results demonstrated experimentally that t_R affects the duration of *in vivo* drug-target binding. The success of studying compounds with a relatively stable PK-$t_{1/2}$ (≥6 h) led us to further explore the possibility of studying a compound that is metabolically unstable. This also allows us to test whether the PK-$t_{1/2}$ affects the duration of *in vivo* drug-target binding. Therefore, APAU was tested because it has a much shorter PK-$t_{1/2}$ (1.4 h) but relatively long sEH-$t_{1/2}$ (31.3 min). Our results showed that a significant second displacement peak was observed in this assay even with the compound that has a short PK-$t_{1/2}$ (Figure 3a). However, the AUC of the second displacement peak of APAU does not correlate well with the sEH-$t_{1/2}$ of the APAU. If the AUC in the PK profile is mainly affected by absorption and elimination of the drug, we hypothesized that the AUC of the second displacement peak from the *in vivo* displacement assay is affected mainly by elimination of the sEH inhibitor (PK-$t_{1/2}$). This is because the second displacement peak corresponds to the *in vivo* amount of sEH-bound inhibitor but not from external administration of the inhibitor. Therefore, once the AUC of the second displacement peak is normalized by the PK-$t_{1/2}$ of the same compound, the normalized AUC of the second displacement peak is affected significantly by the t_R of the inhibitor which dictates the total *in vivo* amount of sEH-bound inhibitor, as we mentioned in the previous experiment. Our data (Figure 3b) show that the normalized AUC of the second displacement peak correlates well with the sEH-$t_{1/2}$ of the inhibitors. All results imply that the t_R plays a very important role in determining the duration of *in vivo* drug-target interaction (target occupancy). A similar result was also observed in rat studies where a significant difference of AUC of the second displacement peak was found between inhibitors with different sEH-$t_{1/2}$ and potency values (Table S1, Figure S11).

Drug-Target Residence Time Affects the PK Profile of the Inhibitor. Having demonstrated that TPPU remains selectively bound to sEH after a long postdosing period *in vivo*, we hypothesized that t_R of the sEH inhibitor modulates the relative duration of drug-target interaction over drug-nontarget interaction. One such interaction is with drug metabolizing enzymes. This tight binding to the target protein will affect the metabolism of the inhibitor and, therefore, affect its PK profile and biological activity. To test this hypothesis, we compared the PK profile of TPPU (t_R = 20 min) and TAPU (t_R = 6 min) between WT and sEH knockout mice. The results should indicate the effect of t_R on the PK profile of the sEH inhibitor, which ultimately affects the in vivo activity of the inhibitor.

As shown in Figure 4a and Figure S12, the PK profile of TPPU in sEH knockout mice is different from that of WT mice, particularly after 24 h. Our data show that the TPPU was fully eliminated after 72 h in sEH knockout mice, while a trace amount of TPPU remained in blood even after 14 days in WT mice. In addition, unlike the PK profile of TPPU in sEH knockout mice, the PK profile of TPPU in WT mice displays a triexponential decline with a terminal phase that persists longer than 14 days (336 h) (Figure S1), while the PK profile of TPPU in sEH knockout mice follows a biexponential decline. Interestingly, the PK profiles of TPAU are not substantially different between WT and sEH knockout mice. TPAU is fully eliminated in the blood within 144 h in sEH knockout mouse, while TPAU is fully eliminated within 168 h in the WT mice without a long terminal phase (Figure 4b and Figure S12). Because the most significant difference among all the *in vitro* parameters between TPPU and TAPU is the sEH-$t_{1/2}$ (Table 1), our results suggest that the t_R of the inhibitors also modulates the PK profile of the inhibitor by protecting it from metabolism and excretion. The studies described above are facilitated by the high potency of the sEH inhibitors, which allow doses to be used that are unlikely to saturate and compete for metabolic pathways.

DISCUSSION

Drug-target residence time (t_R) is proposed to be a key *in vitro* parameter that can complement the common potency measurements such as K_i, K_d, IC50, and EC50, to predict the *in vivo* efficacy of a drug.[4−6,17,30] This is because t_R is a kinetic parameter of a drug that is independent of enzyme and drug concentration and closely resembles an *in vivo* environment where equilibrium is never reached. In addition, even when a drug is bound to its target, in reality, it constantly associates and dissociates from the target. Therefore, in an open system, a kinetic parameter such as t_R is more important. Although numerous previous studies showed that compounds with a long t_R have better *in vivo* pharmacological activity in animal models, these studies rarely demonstrate that compounds with a long t_R prolong the drug-target binding *in vivo*.[13,14,16,18,31−35] Thus, it was very difficult to predict the impact of t_R on *in vivo* efficacy of a drug. For example, one would expect the t_R should have a minimal effect on the drug which has an elimination half-life longer than the t_R.[36] This observation is because the concentration of a drug *in vivo* should dictate the ultimate pharmacological activity of a drug when it is above the binding affinity of a drug. Therefore, the concentration should be the major factor that drives the *in vivo* activity. However, the t_R measured with diluted enzymes *in vitro* do not address the possibility of a rebinding mechanism which can further extend the *in vivo* duration of drug-target binding. This is because most of the *in vitro* kinetic assays are optimized to use a dilute solution to minimize this possibility. However, our data suggest that the rebinding mechanism should be considered, and this is a topic we will address later. In addition, the t_R has a significant impact on the relative accessibility of endogenous substrates to the enzyme even though the t_R is significantly shorter than the PK-$t_{1/2}$. For example, when the t_R of the drug is longer than the rate of substrate binding, it affects the biological outcome significantly.[30] On the other hand, one would expect a covalent inhibitor to have infinite *in vivo* activity on target. However, Schwartz et al. reported that the drug-target residence time of the reversible interaction of covalent inhibitors is equally if not more important to determine the potency of the covalent inhibitors.[37] For example, the reactivation rates for acetylcholinesterase following kinetically irreversible inhibition by *N*-methyl carbamate insecticides is far faster that the t_R values reported here.[38] We will further discuss how our data can address the differences between covalent and reversible inhibitors. Besides, in most cases, modification of t_R, which is calculated from k_{off}, changes the drug binding affinity (K_d or K_i) because K_d is a ratio of k_{off} over k_{on}. Therefore, an approach that can experimentally show the effect of t_R on the duration of drug-target binding could shed light on how t_R affects the *in vivo* activity of a drug. With the technologies developed from previous research and this study, we are able to tackle some of

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the limitations in recent studies regarding drug-target residence time. This will be discussed further in the following paragraphs.

One of the limitations of the studies on drug-target residence time is the availability of techniques to study *in vivo* drug-target binding which often refers to target occupancy. Recent developed technologies, like fluorescence, surface plasma resonance, isothermal titration calorimetry, and biolayer interferometry, make determination of *in vitro* t_R accessible.[7,24,39,40] However, these technologies are not compatible for *in vivo* studies. In order to experimentally probe the duration of drug-target interaction *in vivo*, we developed an *in vivo* displacement assay that allows us to estimate the amount of target-bound drug at different postdosing times. As a proof of concept, sEH was used as a biological model because the tools developed for research on the sEH to control inflammation, pain, and other clinical targets include a library of inhibitors, recombinant sEH from different species, a sEH knockout mouse, sensitive analytical methods for the inhibitors, and a high-throughput screening assays to fully validate this study. TPPU was used as our model compound because of its wide use in research, potency, long t_R, and *in vivo* stability. As shown in Figure 2a, even at postdosing day 7, a very strong second displacement peak of the TPPU was immediately observed in the blood after administration of a high dose of TCPU. Our results with the sEH knockout mouse and weak inhibitors also indicated that this strong second displacement peak corresponds largely to the amount of sEH inhibitor specifically bound to sEH *in vivo* but is not due to nonspecific binding to other proteins (Figures 2a and S3). The substantial second displacement peak observed in this *in vivo* displacement assay could be a result of a high abundance of sEH (the total pool of sEH in mice is estimated to be 10 nmol based on the determined sEH amount (Figure 2C) in several key sEH expressing organs which we assume to account for >80% of sEH in mouse). This amount of sEH should be equivalent to the maximum amount of sEH inhibitor that can be displaced, particularly in liver (1 μmol/kg of liver tissue) which leads to a strong displacement peak being observed.

To understand the importance of t_R alone on *in vivo* duration of drug-target binding, we used a class of sEH inhibitors, where their K_i is similar but significantly different than their t_R. This series of sEH inhibitors helped us to dissect the importance of t_R while minimizing interference from K_i or K_d. With these tools, we demonstrated experimentally the importance of t_R on the duration of *in vivo* drug-target binding. Compounds with long t_R bind to the *in vivo* target with extended residence (Figures 3, S13−S16). As Dr. Paul Ehrlich stated, "corpora non agunt nisi" ("a substance will not work unless it is bound"). The extended duration of drug-target binding leads to a prolonged biological effect which enhances *in vivo* efficacy. Our results showed that t_R affects the*in vivo* activity of drugs through prolonging the *in vivo* drug-target interactions. Because the data on actual target occupancy throughout the dosing period are very limited or absent, the data obtained, and the method developed in this report, could very well be the first step in comprehending and modeling the relationship between t_R and the *in vivo* target occupancy.

While studies show that t_R plays an important role in the *in vivo* activity of a drug, as discussed previously, there is still controversy regarding the relative importance of t_R and PK-$t_{1/2}$ on drug efficacy.[36,41] In this report, to our surprise, the inhibitor still binds to the sEH at postdosing day 7 which is 504-fold longer than t_R of the sEH inhibitors determined from *in vitro* studies in a dilute solution. This result is due to the existence of a drug rebinding mechanism as Vauquelin et al. also suggest in their studies.[42,43] Particularly, we found that a significant amount of sEH is expressed in mammals, especially in the liver (~1 μmol/kg, See Supporting Information Figures 2c, S2 and S17). Therefore, *in vivo* t_R can last longer than the one found *in vitro*. This result also implies that unlike other studies, k_{obs}, which is the product of k_{on} and target protein concentration, should also be included if one wants to accurately translate the *in vitro* potency to *in vivo* efficacy because the rebinding mechanism plays an important role as to how long a drug will stay bound to the target protein. These observations also indicate that the *in vivo* activity of a drug which translate to its *in vivo* efficacy could also greatly be affected by the local concentration of the target protein as one suggested by van Waterschoot et al. especially in our case in which we estimate the concentration of sEH in liver is around 1 μmol/kg or μM.[44]

Although this assay was specifically developed using sEH as a sole biological system, on the basis of our knowledge obtained over decades of sEH research, we believe this assay can also be applied to other therapeutic targets upon fulfilling the following criteria, and we separate the criteria into target-related and technical related. For target-related requirements: (1) Relatively high levels of target protein *in vivo* are needed. A high level of target in tissue ensures a significant amount of drug is bound; thus, the amount of displaced drug is large enough to be detected easily. In addition, a high level of target also facilitates the binding of ligand because drug-target binding is a bimolecular reaction. As a result, it will also facilitate the rebinding mechanism of the drug dissociated from the target which is another important factor, and we will discuss in a later paragraph. (2) The target is expressed in well-perfused tissue. This facilitates the ligand binding to the target and also facilitates the bound ligand being displaced easily during the assay period. (3) A similar rate of synthesis and degradation of the enzyme is needed because the level of the target affects the amount of ligand bound to the target, which affects the amount of displaced the ligand. A stable *in vivo* level of the target throughout the assay period will, therefore, minimize the artifact of the assay results from the increase or decrease of the *in vivo* level of the target. However, if the binding of the ligand affects the rate of either synthesis or degradation of the target, one can determine the maximum target occupancy after the experiment and use this information to normalize the data. For technical requirements: (1) A potent displacement ligand with good bioavailability is needed to ensure that the ligand is potent and highly available *in vivo* to displace the tested ligand that is bound to the target *in vivo*. (2) A sensitive detection method for the tested ligand is required because this will allow us to determine the level of the displaced ligand. However, we do anticipate the detection method will be no longer a limitation in the near future because the technologies for monitoring a drug *in vivo* has been improved substantially over the last decades. For example, with accelerator mass spectrometry, we can detect the drug at the attomole level. In addition, once the technology of detecting compounds *in vivo* is no longer a limitation, we can then apply this assay to a less abundant target.

We also believe this assay can be further extended to other clinically relevant species and ultimately to humans as long as it fulfills the above-mentioned criteria for the assay. However,

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there are several limitations that should be considered: (1) The enzyme from different species may have different selectivities toward the inhibitors. The sEH is one of the examples at a large variation in potency among species. Therefore, we should measure the *in vitro* parameters with the enzyme or receptor from the target species.[24,45−47] (2) The metabolism between species could be different, which affects the bioavailability of the tested compounds. (3) The safety concern of using a high concentration of a displacement compound in clinically relevant species and humans. To circumvent these limitations, one can apply this displacement assay using human and rodent primary cells in which the target enzyme is stably expressed; unfortunately, there is no cell line that stably expresses sEH at levels consistent with *in vivo* expression. Alternatively, one can conduct an *in vivo* displacement assay in rodents followed by obtaining *in vitro* parameters from *in vitro* assays in both rodent and target species. Then, one can use advanced PK modeling to extrapolate the rodent data to predict target occupancy of the drug candidates in humans. In this case, the *in vivo* displacement assay in rodent will help us to build a PK model to determine the impact of t_R and other factors on target occupancy. The data from *in vitro* assays in both human and rodent enzymes or tissues such as K_i and t_R of the inhibitors, *in vitro* metabolism, and the relative expression levels of the target, allow us to extrapolate the rodent data to predict human target occupancy. Our laboratory is currently conducting both studies and will report the results soon. We believe this relatively noninvasive *in vivo* displacement assay could also be applied widely to other pharmaceutical targets as well as to environmental toxicants as long as they fit the above-mentioned criteria. This will also help us to investigate other biological phenomena. For example, while it has been shown that prolonged inhibition of sEH inhibitor does not affect the sEH level in rodents,[48] one could use this technique to investigate whether extended drug-target binding affects the *in vivo* level of the other target over time, which ultimately affects the dose of the ligand for chronic treatment. One could also combine advanced PET scanning technology with the *in vivo* displacement assay to investigate (1) whether the distribution of the drug is due to binding of target or nonspecific binding; (2) estimate the in vivo target enzyme distribution; and (3) determine, for example, the blood−brain barrier penetration.

Much to our surprise, there is only limited recent research into the impact of t_R on the PK profile of a drug. With the sEH knockout mice and sensitive analytical methods, we were able to demonstrate that t_R also affects the *in vivo* activity of the drug through modulating its PK profile. By comparing the PK profile between the WT and sEH knockout mice of the same inhibitor, we have shown that the binding on the target enzyme *in vivo* clearly affects the rate of elimination and metabolism of the sEH inhibitor (Figure 4). In addition, our data show that the difference in PK profiles between WT and sEH knockout mice becomes more significant when the drug has a long t_R. As Callan et al. indicated, t_R also affects the kinetic selectivity of the drug interacting with pharmacological proteins including nontarget-related binding. For example, the duration of drug interacting with target will impact the duration of drug metabolizing by metabolic enzymes.[49] Our results showed that the extended duration of drug-target binding *in vivo* protects the drug from metabolism because it minimizes the binding of the drug by other proteins such as metabolic enzymes or by excretion. This could explain why some compounds or environmental contaminants have an unexpectedly long PK elimination half-life. This finding will help us better understand how t_R affects PK profile of the drug which affects its *in vivo* activity from a different perspective.

The results reported here also address whether a covalent/irreversible inhibitor has significant advantages over a reversible inhibitor. The irreversible or covalent inhibitors which have infinite drug-target residence time are believed to have prolonged biological effects and therefore could potentially lower the dosing regimen and the dosage which is generally considered as important advantages and lower toxicity.[50,51] For example, in patients, clopidogrel maintains the effect beyond 24 h until 7 days even when the majority of the clopidogrel is cleared within 6 h from dosing. Because of this unique property, clopidogrel is often administered with a high loading dose followed by a much lower maintenance dose.[52] However, this does not apply to all covalent inhibitors. For example, several (Bruton tyrosine kinase) Btk irreversible inhibitors have been developed and used to treat several specific cancers.[53−56] It was reported that the dosing frequency for these inhibitors is not well predicted based on the kinetics of inactivation of the target.[10,53] Several factors are suggested to contribute to this outcome. One of the most important factors could be the turnover rate of the target. Unlike reversible inhibitors, where once the target which the inhibitor covalently binds to is degraded, the bound inhibitor can no longer be regenerated. In this case, if the turnover rate of the target is fast, the target occupancy will decrease significantly over time; thus, a repeat dose is needed. This phenomenon has also been shown in irreversible EGFR inhibitors.[57] Therefore, the rate of target synthesis and degradation should be included in the prediction of a dosing regimen for the irreversible inhibitor.[53,58] Therefore, as we showed, the reversible inhibitors would be beneficial because the dissociated inhibitor can, in fact, rebind to the newly synthesized target, and it can maintain the binding to the target *in vivo* much longer than the measured drug-target dissociation half-life because the target acts as a refectory pool of the inhibitor *in vivo*. Once the inhibitor is released, it will bind to the newly synthesized target in vivo. Unfortunately, in this study, we cannot test whether inhibitors with a higher target occupancy at day 7 are more potent than the inhibitor with less target occupancy because sEH is a low vulnerability target[58] where high inhibition of the sEH (≥90%) is required for inhibitors to show the effect.[29] However, we have recently published studies to demonstrate that the inhibitors with a long t_R are more potent and have extended *in vivo* activity, but the limitation in these studies is we have not considered the PK profiles of the tested inhibitors.[24,59] In fact, we are currently testing this assay at earlier time points where 99% of sEH is inhibited and will use this assay to study in detail the effects of inhibitors' PK profile, t_R, target occupancy time profile on their activity *in vivo*.

How t_R influences the pharmacology of drug action of course depends in part upon the biology of the system. This topic is not addressed in detail here; however, it is clear that the t_R of inhibitors of the sEH will be major factors influencing the epoxide hydration and inactivation of the epoxy-fatty acids. Most of these substrates are bioactive at low nanomolar to picomolar concentrations and are degraded with a high k_{cat}/K_m ratio. Under such conditions, one expects the t_R to be an important predictor of *in vivo* activity because the high k_{cat}/K_m ratio is dominated by the low K_m term coupled with the very low *in vivo* abundance epoxy-fatty acid substrates. Thus, even small amounts of uninhibited sEH could have a large impact

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on the titer of epoxy-fatty acids. One would expect t_R to have a greater impact on sEH biology than on acetylcholine esterase, which also metabolizes a chemical mediator with a high k_{cat}/K_m ratio. However, the k_{cat}/K_m ratio is dominated by a high k_{cat}, and its substrate acetylcholine is present at a relatively high concentration within the synaptic cleft. Therefore, the effect of t_R on the biology of the system will be context dependent.

In summary, we generated a new assay that facilitates estimation of the amount of target-bound compound *in vivo*. This assay allows us to study how t_R affects duration of *in vivo* target binding in a relatively easy manner. Although one may argue that it could be difficult to address other drug candidates with an analogous assay because of a low receptor level or target protein, mass spectrometry technology has been significantly improved during the last few decades and improvement continues. Alternately, one can either use positron emission tomography or a C14 mass label with accelerator mass spectrometry to substantially enhance sensitivity to detect the displaced low levels of compounds. This assay could also potentially be used for the exploration of different pharmacological phenomena; for example, the in vivo displacement assay could be used to address the different pharmacological activities of drugs among species.

Most importantly, the results presented in this study clearly demonstrate the effects of t_R on the duration of target binding. Our data also suggest that t_R affects its drug efficacy *in vivo* not only through target interaction but also through modulating the PK profile of the drug, which, to our knowledge has not been approached to date. Although one may argue the success of this study is mainly because sEH is well-expressed *in vivo*, as we continue improving the potency of the drug candidates which also improves the t_R, our findings will be able to be applied to other drug targets. In fact, more PK studies and *in vitro* studies have reported similar observations with small molecules, which sometimes refer to a phenomenon called target-mediated drug disposition (TMDD).[44,60−62] In addition, our study provides important data on looking at the unclear relationship between t_R, the duration of *in vivo* target binding and PK profile, as well as the factors that could affect the contributions of t_R to its *in vivo* activity that translate to its efficacy. The data generated from this study and the assay described will help to better model and predict drug efficacy using data obtained from *in vitro* studies as well as *in vivo* animal studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.9b00770.

Supporting Figures S1−S19, Supporting Tables S1−S3, Experimental methods and materials (PDF)

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Notes

The authors declare the following competing financial interest(s): The University of California holds patents on the sEH inhibitors to treat disease. Some of these patents have been licensed by EicOsis L.L.C., a startup company advancing sEH inhibitors as potential therapeutics. BDH is cofounder and JY and KW are employees of EicOsis L.L.C.

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