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

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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_d, K_I, IC_{50} or EC_{50}) and is a better predictor of in vivo efficacy. Compounds with a long drug-target residence time in vitro are 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.6−8 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.3−6 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_{sof}, 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,
blood pressure, pain perception, and angiogenesis, which makes it a good drug target. 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 Ki and tR of the inhibitors of sEH that have recently been established; 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 bound drug 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 (Cmax) that is at least 100 times above the Ki 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.

**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 tR 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 (Cmax) that is at least 100 times above the Ki 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-
(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. 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 $\ln 2 \ast t_R$ 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) = $56254 \, nM \cdot h$, Table 1). These properties ensure that the sEH-bound TPPU can be displaced by a high in vivo concentration of TCPU. 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 ($\sim 182$-fold of $K_i$) and AUC at 8813 nM·h. In addition, this dose of TPPU has demonstrated pharmacological activity in several animal models. 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. 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 ($\sim 15 \, nM$) 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. Here we hypothesize that there is a significant amount of TPPU remaining in vivo and mostly

<table>
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<th>Structure</th>
<th>$^a$ LogP</th>
<th>$^a$ $K_i$ (nM)</th>
<th>$^a$ sEH-$t_{1/2}$ (min)</th>
<th>$^b$ In vitro Plasma to Blood Ratio$^c$</th>
<th>$^d,e$ Dose (mg/kg)</th>
<th>$^d,e$ AUC (nM$^*$h)</th>
<th>$^d,e$ $C_{max}$ (nM)</th>
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<td>N.D.$^f$</td>
<td>N.D.$^f$</td>
<td>3</td>
<td>1504</td>
<td>1019</td>
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$^a$LogP, $K_i$ and $t_{1/2}$ were reported before, and $K_i$ and $t_{1/2}$ were measured with murine sEH. The results are the average of triplicates with ± SEM. $^b$Plasma protein binding (%) was reported by Lee et al. $^c$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: $\text{logP}$ stands for experimental log P which was determined by the HPLC method in Lee et al.
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 \textit{in vivo} level of TPPU is stable after postdosing day 7 (Figure 2a), any subtle changes of the time of 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 S1. (b) The result from the \textit{in vivo} displacement assay in rat where the TCU (0.1 mg/kg in PEG400/oleate 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/oleate 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/oleate 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 \textit{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 2. (a) The \textit{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 \textit{in vivo} displacement assay in rat where the TCU (0.1 mg/kg in PEG400/oleate 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/oleate 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/oleate 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 \textit{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. $t_{f_0}$ 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 $t_{f_0}$ of the tested inhibitor in the \textit{in vivo} displacement assay. The AUC of second peak correlates well with inhibitors $t_{f_0}$ 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 $t_{f_0}$ 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 administered 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 \textit{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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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 of the same 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-1/2 (which implies the same as $t_{1/2}$) 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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**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 detection level 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.

(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.
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 \( t_\text{1/2} \) affects the duration of in vivo drug-target binding. The success of studying compounds with a relatively stable PK-\( t_\text{1/2} \) (≥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-\( t_\text{1/2} \) affects the duration of in vivo drug-target binding. Therefore, APAU was tested because it has a much shorter PK-\( t_\text{1/2} \) (1.4 h) but relatively long sEH-\( t_\text{1/2} \) (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-\( t_\text{1/2} \) (Figure 3a). However, the AUC of the second displacement peak of APAU does not correlate well with the sEH-\( t_\text{1/2} \) 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-\( t_\text{1/2} \)). 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-\( t_\text{1/2} \) of the same compound, the normalized AUC of the second displacement peak is affected significantly by the \( t_\text{1/2} \) 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-\( t_\text{1/2} \) of the inhibitors. All results imply that the \( t_\text{1/2} \) 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-\( t_\text{1/2} \) 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 \( t_\text{1/2} \) 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 (\( t_\text{1/2} = 20 \) min) and TAPU (\( t_\text{1/2} = 6 \) min) between WT and sEH knockout mice. The results should indicate the effect of \( t_\text{1/2} \) 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-\( t_\text{1/2} \) (Table 1), our results suggest that the \( t_\text{1/2} \) 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 vivo parameter that can complement the common potency measurements such as \( K_i, K_d, IC_{50}, \) and EGS0, to predict the in vivo efficacy of a drug.3,5,6,17,30 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.3,5,14,16,18,31–35 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-\( t_\text{1/2} \). 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.35 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 \( k_{\text{off}} \) changes the drug binding affinity (\( K_i \) or \( K_d \)) because \( K_i \) is a ratio of \( k_{\text{off}} \) over \( k_{\text{on}} \). 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...
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 $t_b$ accessible. 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 $t_b$, 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 the 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 $\mu$mol/kg of liver tissue) which leads to a strong displacement peak being observed.

To understand the importance of $t_b$ 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 $t_b$. This series of sEH inhibitors helped us to dissect the importance of $t_b$ while minimizing interference from $K_i$ or $K_d$. With these tools, we demonstrated experimentally the importance of $t_b$ on the duration of in vivo drug-target binding. Compounds with long $t_b$ 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 $t_b$ affects their 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 $t_b$ and the in vivo target occupancy.

While studies show that $t_b$ plays an important role in the in vivo activity of a drug, as discussed previously, there is still controversy regarding the relative importance of $t_b$ and $PK_{f_{1/2}}$ on drug efficacy.30,31 In this report, to our surprise, the inhibitor still binds to the sEH at postdosing day 7 which is 504-fold longer than $t_b$ 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 $\mu$mol/kg. See Supporting Information Figures 2c, S2 and S17). Therefore, in vivo $t_b$ can last longer than the one found in vitro. This result also implies that unlike other studies, $K_{d,obs}$ which is the product of $K_{d}$ 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 translates 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 $\mu$mol/kg or $\mu$M.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,
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 tR and other factors on target occupancy. The data from in vitro assays in both human and rodent enzymes or tissues such as Km and tR 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 tR on the PK profile of a drug. With the sEH knockout mice and sensitive analytical methods, we were able to demonstrate that tR 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 tR. As Callan et al. indicated, tR 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 tR 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.50,51 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 cannot 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 target58 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 tR 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, tR, target occupancy time profile on their activity in vivo.

How tR 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 tR 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 kcat/Km ratio. Under such conditions, one expects the tR to be an important predictor of in vivo activity because the high kcat/Km ratio is dominated by the low Km 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.
on the titer of epoxy-fatty acids. One would expect \( t_k \) to have a greater impact on sEH biology than on acetylcholine esterase, which also metabolizes a chemical mediator with a high \( k_{out} / K_m \) ratio. However, the \( k_{out} / K_m \) ratio is dominated by a high \( k_{out} \) and its substrate acetylcholine is present at a relatively high concentration within the synaptic cleft. Therefore, the effect of \( t_k \) 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 \( t_k \) 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 sensitivity to detect the displaced compounds.


target-mediated drug disposition (TMDD).44,60

Most importantly, the results presented in this study clearly demonstrate the effects of \( t_k \) on the duration of target binding. Our data also suggest that \( t_k \) 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 \( t_k \), 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 \( t_k \) and the duration of in vivo target binding and PK profile, as well as the factors that could affect the contributions of \( t_k \) 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 co-founder and JY and KW are employees of EicOsis L.L.C.

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