Published in final edited form as:

J Am Chem Soc. 2017 May 31; 139(21): 7132–7135. doi:10.1021/jacs.7b00962.

Rational Optimization of Mechanism-Based Inhibitors through Determination of the Microscopic Rate Constants of Inactivation

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Abstract

Mechanism-based inhibitors (MBIs) are widely employed in chemistry, biology, and medicine due to their exquisite specificity and sustained duration of inhibition. Optimization of MBIs is complicated due to time-dependent inhibition resulting from multi-step inactivation mechanisms. The global kinetic parameters k_{inact} and K_{I} have been used to characterize MBIs, but provide far less information than is commonly assumed, as shown by derivation and simulation of these parameters. We illustrate an alternative and more rigorous approach for MBI characterization through determination of the individual microscopic rate constants. Kinetic analysis revealed the rate-limiting step of inactivation of the PLP-dependent enzyme BioA by dihydro-(1,4)-pyridone **1**. This knowledge was subsequently applied to rationally design a second-generation inhibitor scaffold with a nearly optimal maximum inactivation rate (0.48 min^{-1}).

Graphical abstract

Mechanism-based inhibitors (MBIs) are unreactive molecules that, through enzymatic catalysis, are transformed into an active species that inhibits the enzyme, typically through covalent modification of the active site.^{1,2} MBIs represent the ultimate class of enzyme

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Supporting Information.

Experimental details and additional data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.xxxxxxx.

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Notes

The authors declare no competing financial interest.

inhibitors for drug development due to both sustained inhibition through the aforementioned covalent interactions as well as their extremely high potential for selectivity and specificity.^{3–5} The initial binding event provides selectivity for an individual enzyme while the activation step engenders an additional level of specificity toward enzymes catalyzing similar chemistry. As a result, MBIs are widely used in medicine, accounting for over 50 marketed drugs⁶ with numerous development efforts ongoing.^{7,8}

Unlike reversible inhibitors where one can improve potency through enhancing the ground-state binding energy (ΔG), optimization of MBIs requires increasing the microscopic rate constants of inactivation. As these parameters can be challenging to obtain, many campaigns instead aim to increase the parameter $k_{\text{inact}}/K_{\text{I}}$, where k_{inact} is the maximum rate constant of inactivation achievable, and K_{I} is the concentration of inhibitor producing half of k_{inact} .² For a two-step inactivation mechanism with a rapidly reversible first step (common for affinity labels), these parameters are quite informative, as K_{I} correlates with the dissociation constant (K_{D}) of the initial binding event, and k_{inact} is the rate of formation of the fully inactivated complex (Table 1). In this case, $k_{\text{inact}}/K_{\text{I}}$ accurately measures the activity of an MBI, similar to how an enzyme's $k_{\text{cat}}/K_{\text{M}}$ value conveys its efficiency.^{2,5} In more common inactivation mechanisms involving greater than two steps, k_{inact} and K_{I} become complex aggregates of rate constants (Table 1) that no longer correlate to a particular step. (See Table S1 for description and derivation of several alternate mechanisms). Specifically, reversible steps beyond the initial binding event decouple k_{inact} from the rate limiting step (Table 1, S1, & S2), and only correlates with K_{D} if the step directly following binding is both irreversible and entirely rate limiting (Table S2). Therefore, determination of k_{inact} and K_{I} is much less informative, and utilizing $k_{\text{inact}}/K_{\text{I}}$ to guide optimization attempts does not necessarily provide an accurate assessment of the potency of an MBI. A more effective method is thus necessary for MBI development, especially towards guiding future synthetic chemistry efforts. We propose that determination of all of the individual rate constants of inactivation would furnish a complete profile of an MBI, providing several advantages to investigators. This profile includes not only the identity of the critical rate-limiting step(s), but also an accurate measure of binding affinity for an MBI. It would also allow for identification of reversible steps subsequent to binding that, if improved, produce a multiplicative enhancement of potency of inhibition (Table S2). This would greatly inform the synthesis of new MBIs that are specifically designed to target the key step(s).

The most popular targets for MBI development have been pyridoxal phosphate (PLP)-dependent enzymes, a common class of enzymes for drug development due to the extraordinary breadth of chemistry they can catalyze.⁹ Their catalytic cycle involves removal of a proton, facilitating activation of many chemical entities.^{1,3} We previously described dihydropyridone **1** as an MBI of BioA,¹⁰ a PLP-dependent aminotransferase that conditional knockdown experiments identified as essential in *Mycobacterium tuberculosis*.¹¹ Given the presumed four-step mechanism of inactivation (Figure 1A), it was unclear how to further optimize **1** based on the obtained K_{I} and k_{inact} values. We therefore selected BioA and MBI **1** as a model system and herein describe the complete characterization of the system followed by application of the knowledge to rationally design an inhibitor with an improved kinetic profile.

PLP-dependent enzymes undergo a series of states with distinguishable absorbance signatures, ideal for study with stopped-flow spectrophotometry. Incubation of **1** with BioA under stopped-flow conditions utilizing a diode-array detector showed three distinct absorbance regions with large, time-dependent changes (Figure 1B). An absorbance peak with a λ_{max} of 410 nm decreases over time, while a corresponding increase is observed at 318 nm. These changes are likely caused by disappearance of the external aldimine and subsequent formation of the final, aromatized adduct (Figure S1). Finding a peak with a λ_{max} of 540 nm, which increases and then decreases in magnitude (Figure S2), can be surmised to be due to the presence of a quinonoid.⁹ Previous examination of quinonoid intermediates in PLP-dependent mechanisms generally place the λ_{max} between 490 and 520 nm, though observation of such species is quite rare.¹² We propose that the additional double bond on **1** that is in resonance with the quinonoid (Figure 2A) causes the observed red shift of the peak.

Fitting the reaction time course observed at 540, 410, or 318 nm to summed exponential expressions revealed two fast and one slow reciprocal relaxation times (RRTs) (Figure S1, S2). The slow RRT had a value significantly less than the k_{inact} , indicating that it arises a minor side reaction. Therefore, kinetic evidence for only two of the four postulated steps was initially obtained. However, close inspection of the earliest time points in the 540 nm time course showed a very short (<2 s) burst in absorbance that was previously unaccounted for (Figure S2). Refitting the 540 nm time course to include another exponential with a very fast RRT allowed the early time domain to be accurately modeled. The observation of three kinetically competent RRTs shows that the reaction must have at least 3 steps.

An examination of the concentration dependence on **1** for each of the kinetically competent RRTs reveals distinctive patterns. The fastest RRT displays a linear correlation with the concentration of **1**, while the second fastest RRT shows no dependence and the slowest RRT has a hyperbolic dependence (Figure 2A–C). The magnitudes of the RRTs are separated by ~30-fold, facilitating the data analysis. The linear relationship for the fastest RRT means that it reflects primarily binding of **1**, probably through rapid formation of the external aldimine. The very slight absorbance increase at 540 nm is unexpected for the aldimine, but feasible.^{9,12} The intercept and slope of the plot give the reverse and forward rate constants for binding, respectively, while the ratio gives the binding K_D . Because the reaction is reversible and the RRT is large, it is likely that this binding reaction reaches near equilibrium prior to the downstream reactions. If this is the case, then one would expect the plot of at least one of the other RRTs to exhibit a hyperbolic concentration dependence on **1**, as seen for the slowest RRT (Figure 2C). The forward and reverse rate constants for the step following initial complex formation, as well as the K_D for the immediately preceding binding reaction can be computed from the hyperbolic curve (see SI). Accordingly, the K_D computed in this way is in good agreement with the K_D computed from the plot in Figure 2A (180 vs 240 μM). The intercept at zero of the hyperbolic curve means that the remaining RRT should not exhibit a concentration dependence on **1** and gives information about only the rate constants for downstream steps. Thus, the lack of concentration dependence on **1** of the second fastest RRT (Figure 2B) indicates that it correlates with the rate constant(s) for a step or steps that follow the irreversible second step of the sequence. This analysis suggests

that the slowest step in the sequence is the formation of the quinonoid, and the second fastest step corresponds to its disappearance.

The microscopic rate constants of inactivation are displayed in Figure 2D. Using the formula from Table 1, entry 2, K_I and k_{inact} were calculated from the determined rate constants and are within 2-fold of the experimentally determined values under steady-state conditions (Table S5). This assignment of rate constants to specific steps was also confirmed by multiple checks using simulation and estimation of the maximum amount of quinonoid formation (Figure S3, Table S4). It is notable that the tautomerization step to form the aromatized adduct is not observed by this analysis, and thus must be significantly ($>30\times$) faster than ketimine formation, which is plausible as its rate would only be limited by the access of the bound inhibitor to proton donors and acceptors.

The kinetic analysis reveals that there are no detectable reversible steps post-binding and that the second step is almost entirely rate-limiting, which means K_I approximates the K_D of the Michaelis complex [BioA•**1**] and k_{inact} approximates the rate of formation of the quinonoid, providing us with the unique opportunity to rationally improve it. Since the quinonoid is formed following the deprotonation of the α -proton on **1** by Lys283 of BioA (Figure 2), we hypothesized lowering the pK_a of this proton would enhance k_{inact} . This was accomplished by synthesis of dihydro-(1,4)-pyridone ($\pm$)-**2**,¹³ a constitutional isomer of dihydro-(1,2)-pyridone ($-$)-**1**, which is calculated to decrease the pK_a of the α -proton approximately 6 units (see Supporting Information). Compound **2** displayed time-dependent inhibition of BioA (Figure S4), and a crystal structure of the final, inactivated PLP adduct with **2** was also obtained (Figure 3 and S6), confirming **2** leads to mechanism-based inhibition of the PLP via aromatization. The K_I and k_{inact} were subsequently determined by plotting the observed rate of inactivation as a function of inhibitor concentration (Table 2) to determine whether we successfully raised the rate of deprotonation, as any increase in k_{inact} would have to be from an improvement in the rate of deprotonation (Table S2). Dihydro-(1,4)-pyridone **2** possesses a nearly 3-fold increase in k_{inact} relative to **1**, validating our design strategy. The k_{inact} of **2** is lower than we would have expected based on the pK_a differences between **1** and **2**, but approaches BioA's turnover number (k_{cat}) of $\sim 1.0 \text{ min}^{-1}$ with its native substrate 8-amino-7-oxononanoic acid, indicating **2** has achieved a nearly optimal maximum rate of inactivation. The observed attenuation in K_I demonstrates that substitution of a dihydro-(1,4)-pyridone for a dihydro-(1,2)-pyridone causes a slight loss of binding affinity. However, inhibitor ($\pm$)-**2** was prepared as a racemic mixture, whereas ($-$)-**1** is enantiopure and the (+)-antipode of **1** is biologically inactive, thus the K_I for enantiopure **2** is likely two-fold lower than our reported value. The adduct crystal structure also suggests opportunities to further enhance affinity by modification of the 3-hydroxypropyl side-chain in **2**.

As demonstrated by our analysis, the approach described towards MBI optimization provides complete knowledge of where medicinal chemistry efforts should be focused. We believe that this approach is widely applicable towards most of the classes of enzymes for which MBIs are commonly developed. PLP-dependent enzymes are certainly an ideal class due to the simplicity of spectroscopically monitoring the PLP-cofactor, but this approach could also easily be implemented for flavin-containing enzymes, heme-dependent oxidases,

quinone-dependent oxidases, and cases where the inhibitor itself can be monitored spectrophotometrically. In such cases, complete mechanistic characterization would be both practical and very useful.

Despite nearly fifty years of development, MBI strategies have thus far relied on semi-empirical optimization by measuring the global kinetic parameters k_{inact} and K_I . We have identified a large number of conditions where determination of k_{inact} and K_I provides insufficient information to properly inform future optimization. This can be overcome through complete kinetic characterization of an MBI, which we successfully performed for **1**, an inhibitor of the PLP-dependent enzyme BioA. The resulting kinetic profile was used to rationally improve the maximum inactivation rate of the MBI. Though this approach is time consuming, it provides unparalleled insight into the inhibition process and is pragmatic in a wide variety of systems and enzyme classes that commonly employ MBIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants (AI091790 to C.C.A., GM118030 to J.D.L.) from the National Institutes of Health. C.E. thanks the NIH for a Chemistry-Biology Interface Predoctoral Traineeship (GM08700). We thank Melanie Rogers and Brent Rivard for assistance with the transient kinetic experiments, and Kathleen Wang for assistance with derivations.

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Figure 1. BioA mechanism and observed inactivation by compound **1**. (A) Postulated mechanism of inactivation of BioA by compound **1**. (B) Absorbance traces from incubation of 12.5 μM BioA with 250 μM **1** in BICINE pH 8.6 at 23 $^{\circ}\text{C}$

Figure 2.

(A–C) Dependence of the observed RRTs on the concentration of 1. (D) Rate constants determined from analysis of graphs A–C. First step forward rate constant is the slope in part (A); reverse rate constant is the intercept in part (A). Second step rate constant is the asymptote of the hyperbola in part (C) minus the y-intercept. Third step rate constant is the y-value in part (B).

Figure 3. Omit map (mF_0-DF_0 , contoured at 3.0σ) shows full reaction of **2** with the PLP of BioA. This structure (teal) is compared to the internal aldimine complex (tan; 4CXQ) with bound substrate (not shown).¹³

Table 1Common mechanisms of mechanism-based inhibition and their associated K_I and k_{inact} values.

Entry:	Mechanism:	K_I (M)	k_{inact} (s^{-1})
1	$\text{E} + \text{I} \xrightleftharpoons[k_{-1}]{k_1} \text{EI} \xrightarrow{k_2} \text{EI}^*$	$\frac{k_{-1} + k_2}{k_1}$	k_2
2	$\begin{array}{c} \text{E} + \text{I} \xrightleftharpoons[k_{-1}]{k_1} \text{EI} \xrightarrow{k_2} \text{EA} \xrightarrow{k_3} \text{EA}^* \\ \quad \quad \quad \downarrow k_4 \\ \quad \quad \quad \text{E} + \text{A} \end{array}$	$\frac{(k_{-1} + k_2)(k_3 + k_4)}{k_1(k_2 + k_3 + k_4)}$	$\frac{k_2 k_3}{k_2 + k_3 + k_4}$
3	$\text{E} + \text{I} \xrightleftharpoons[k_{-1}]{k_1} \text{EI} \xrightleftharpoons[k_{-2}]{k_2} \text{EI}^+ \xrightarrow{k_3} \text{EI}^*$	$\frac{k_{-1}(k_{-2} + k_3) + k_2 k_3}{k_1(k_2 + k_{-2} + k_3)}$	$\frac{k_2 k_3}{k_2 + k_{-2} + k_3}$

Table 2Kinetic Parameters for BioA Inactivation.¹⁰

Inhibitor	Structure	k_{inact} (min ⁻¹)	K_{I} (mM)	$k_{\text{inact}}/K_{\text{I}}$ (mM ⁻¹ min ⁻¹)
1		0.18 ± 0.01	0.52 ± 0.07	0.35 ± 0.07
2		0.48 ± 0.12	3.9 ± 1.2	0.12 ± 0.07