Inhibitors

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Rapid Determination of the Specificity Constant of Irreversible Inhibitors (k_{inact}/K_I) by Means of an Endpoint Competition Assay

Ikuo Miyahisa, Tomoya Sameshima, and Mark S. Hixon*

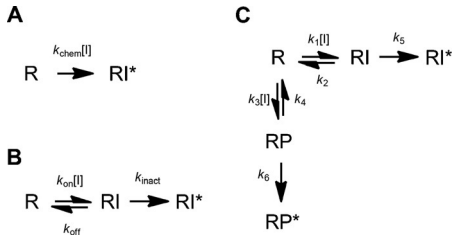
Abstract: *Owing to their covalent target occupancy, irreversible inhibitors require low exposures and offer long duration, and their use thus represents a powerful strategy for achieving pharmacological efficacy. Importantly, the potency metric of irreversible inhibitors is k_{inact}/K_I not IC_{50}. A simple approach to measuring k_{inact}/K_I was developed that makes use of an irreversible probe for competitive assays run to completion against test compounds. In this system, the k_{inact}/K_I value of the test compound is equal to $(k_{inact}/K_I)_{probe} \times [probe]/IC_{50}$. The advantages of this method include simplicity, high throughput, and application to all target classes, and it only requires an in-depth kinetic evaluation of the probe.*

Potent and prolonged interaction of drugs with their targets at low exposures is often critical for successful in vivo efficacy.[1] Irreversible inhibition through covalent addition to the target is arguably the most powerful strategy to achieve this goal since release from inhibition requires resynthesis of the target.[2] Aspirin and penicillin are classic examples of irreversible-inhibitor drugs and more recently omeprazole, clopidogrel, and afatinib have been added to the list of FDA-approved drugs of this type. Collectively, these successes have captured the interest of the pharmaceutical industry.

During lead discovery of reversible inhibitors, a half-maximal inhibitory concentration (IC_{50}) value for ligand displacement (substrates or probes) is commonly employed for the evaluation of inhibition potency. By using the Cheng–Prusoff equation, IC_{50} is converted into an inhibition constant K_i, a quantitative evaluation metric of the potency.[3] Unfortunately, IC_{50} is a poor evaluation metric for test compounds displaying time-dependent inhibition and in particular, irreversible inhibitors.[2] With sufficient time, IC_{50} no longer measures inhibitor potency but instead measures the enzyme concentration employed in the assay. The quantitative potency metric of irreversible inhibitors is the second-order rate constant of target inactivation, k_{inact}/K_I ($M^{-1}s^{-1}$).[2] When compared to the ease of IC_{50} determination, obtaining k_{inact}/K_I for irreversible inhibitors is considerably more laborious, requiring kinetic measurements and complicated curve fitting. Recently, Krippendorff et al. reported a medium-throughput method to extract K_I and k_{inact} for irreversible inhibitors directly from IC_{50}-type assays by conducting the assays at multiple run times. While broadly applicable and certainly more rapid than conventional progress-curve analysis, Krippendorff's method still requires an activity assay, complex curve fitting, and as presented, a minimum of four distinct assay times per compound to obtain reasonable estimates.[4] Since a quantitative evaluation of k_{inact}/K_I for large numbers of inhibitors is critical for lead generation, a more efficient evaluation method is required.

To address this issue, we present a strategy and theoretical background for obtaining k_{inact}/K_I from a competitive binding assay (more precisely an internal-competition assay) by using an irreversible probe. Through a simple kinetic relationship akin to a Cheng–Prusoff equation, k_{inact}/K_I is determined from the typical endpoint IC_{50} assay procedure. The method is compatible with the existing hardware and software used in IC_{50} screens.

There are two commonly encountered kinetic mechanisms of irreversible inhibition. In our notation, R and I represent a drug receptor (or enzyme) and an irreversible inhibitor, respectively. Mechanism A depicts one-step irreversible inhibition, i.e., the inactivation rate is nonsaturating with increasing inhibitor concentration (Scheme 1 A). Mechanism B contains two-steps: in the first step, an inhibitor forms a reversible complex with a receptor (RI) prior to final covalent attachment (RI*; Scheme 1 B). The rate constants for these two mechanistic classes of irreversible inhibitors are k_{chem} and k_{inact}/K_I, respectively.[5]



Scheme 1. Binding processes of one-step (A) and two-step (B) irreversible inhibitors. An asterisk (*) indicates covalently bound form of a receptor. C) Depiction of the probe-competition method. R = receptor, I = inhibitor, P = probe.

In this study, we consider the following system: a probe (P) is irreversible and competitive with the test inhibitors and is observable only when it forms a covalent complex with the target receptor (RP*).[6] For the sake of simplicity, consider

[*] I. Miyahisa, Dr. T. Sameshima
Biomolecular Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd.
26-1, Muraoka-higashi 2-chome, Fujisawa, Kanagawa 251-8555 (Japan)
E-mail: ikuo.miyahisa@takeda.com
Dr. M. S. Hixon
Modeling & Simulation, Global DMPK, Takeda California, Inc.
10410 Science Center Drive, San Diego, CA 92121 (USA)

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a model comprising a two-step binding probe and a two-step inhibitor (Scheme 1 C). At t = 0, a series of assays are initiated by the addition of R to solutions containing a fixed probe concentration but varying concentrations of a test inhibitor. The observable signal arises from RP* and increases with time. The assumptions in this model are; 1) both the probe and inhibitor are irreversible, 2) they bind mutually exclusively to R, 3) they display 1:1 binding to R, and 4) no receptor-mediated ligand depletion occurs. Under our assumptions, R, RP, and RI disappear at infinite reaction time, leaving only RP* and RI* to account for all the receptor molecules present in the initial state. Therefore, the observed signal of the probe-competition assay is proportional to $[RP^*]_i/[RP^*]_0$, where $[RP^*]_0$ and $[RP^*]_i$ indicate [RP*] in the absence or presence of a specific concentration of an inhibitor, respectively. $[RP^*]_i/[RP^*]_0$ equals $[RP^*]_i/[R]_{total}$ at reaction completion. Expressing $[RP^*]_i/[R]_{total}$ as a function of [I] gives a binding isotherm equation, i.e., an IC_{50} equation (see derivation S1 in the Supporting Information) and the relationship between an observed IC_{50} of the assay and evaluation metrics for two-step irreversible inhibitors (k_{inact}/K_I) is expressed as:

$$(k_{inact}/K_I)_{inhibitor} = (k_{inact}/K_I)_{probe} \times [Probe]/IC_{50} \quad (1)$$

In application, the k_{inact}/K_I of the irreversible probe is determined by conventional irreversible-inhibitor kinetic measurements. The experiment provides k_{obs} values for RP* formation, which in turn will determine the half-life for receptor occupation. The probe-derived half-life is used to define the minimum required assay run time. Importantly, the half-life of remaining receptor becomes shorter in the presence of test inhibitors, thus those assays not containing test compound will be the slowest to reach endpoint. Assay sensitivity is greatest at reaction completion, hence, an incubation time to occupy more than 97% of the receptor (5 × half-lives) is desirable.

Our approach was examined by using irreversible inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase (Figure S1 in the Supporting Information).[7] To evaluate the k_{inact}/K_I values of these EGFR inhibitors, we developed a competitive-ligand filter binding assay by using tritiated CI-1033 ([^{3}H]CI-1033) as a probe. CI-1033 (Figure 1 A) binds in the ATP pocket and forms a covalent bond between the acrylamide warhead and Cys797 through Michael addition.[8]

After incubation of [^{3}H]CI-1033 with the enzyme, acetone was added to denature the enzyme and enable us to monitor only the covalently bound RP* enzyme form. Under these conditions, a time-dependent increase in RP* was observed that fitted well to a single-exponential binding equation, thus suggesting 1:1 binding of [^{3}H]CI-1033 to EGFR (Figure 1 B). Progress-curve analysis afforded a k_{inact}/K_I of $(3.5 \pm 0.06) \times 10^5\,M^{-1}s^{-1}$. Irreversible binding of [^{3}H]CI-1033 was confirmed by a dilution experiment (Figure S2 in the Supporting Information), thus establishing [^{3}H]CI-1033 as a suitable competition probe.

Equation (1) predicts that IC_{50} will be linearly proportional to probe concentration, with a slope equal to the ratio

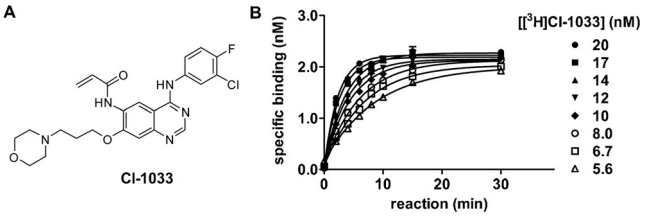

Figure 1. A) Chemical structure of CI-1033. B) Progress curve of RP* formation at varied concentrations of [^{3}H]CI-1033. Data are given as the mean ± SEM ($n = 3$).

of $(k_{inact}/K_I)_{probe}$ to $(k_{inact}/K_I)_{inhibitor}$. We investigated this relationship by examining the shift in dacomitnib (inhibitor) IC_{50} obtained when conducting the competition assays at varied probe concentrations. Dacominitib is a covalent EGFR inhibitor that forms a covalent bond with Cys797.[9] Figure 2 A

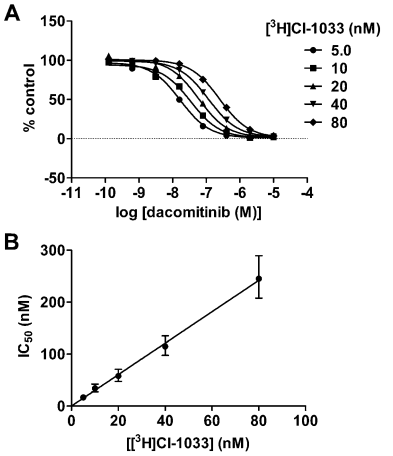

Figure 2. A) Influence of probe ([^{3}H]CI-1033) concentration on dacomitinib IC_{50} in a 60 min assay. Data are given as the mean ± SEM ($n = 3$). B) Replot of the IC_{50} values obtained from (A) versus probe concentration. Data are presented with a 95% confidence interval.

shows that the IC_{50} value of dacomitinib increases with increasing probe concentration. As predicted, the IC_{50} dependence on probe concentration was linear (Figure 2B) and the slope obtained was 3.2, which agrees well with the ratio of probe (**1**) to inhibitor (**2**) k_{inact}/K_I, which was 2.8 (Table 1).

Next, the dynamic range of the method was tested by selecting a range of EGFR inhibitors spanning nearly four orders of magnitude in k_{inact}/K_I. In the presence of 5 nM probe and a gradient of inhibitor concentrations, enzyme occupancy by the probe was monitored (see Note S1 in the Supporting Information). From the IC_{50} values obtained by this assay, the k_{inact}/K_I for each inhibitor was determined by using Equation (1). In a parallel experiment, the k_{inact}/K_I of each inhibitor was determined by conventional kinetic assays (see the Supporting Information). As seen in Figure 3, there is excellent agreement between the k_{inact}/K_I values obtained by the probe-competition assay and conventional kinetic assays. When plotting the results in log–log space, a linear relationship exists with a slope of 0.94. On the other hand, as seen in

Table 1: k_{inact}/K_I values for various EGFR inhibitors [$M^{-1}s^{-1}$].

	Kinetic Measurement	Probe Competition
1, CI-1033	$(1.1\pm0.02)\times10^6$	$(5.2\pm0.3)\times10^5$
2, dacominitib	$(3.9\pm1.0)\times10^5$	$(1.4\pm0.09)\times10^5$
3, WZ-8040	$(1.7\pm0.02)\times10^5$	$(9.6\pm0.6)\times10^4$
4, WZ-4002	$(3.3\pm0.03)\times10^4$	$(1.7\pm0.2)\times10^4$
5, JAK3 inhibitor V	$(9.8\pm0.4)\times10^3$	$(8.2\pm0.8)\times10^3$
6, Ro 106-9920	$(3.6\pm0.13)\times10^2$	$(2.0\pm0.2)\times10^2$

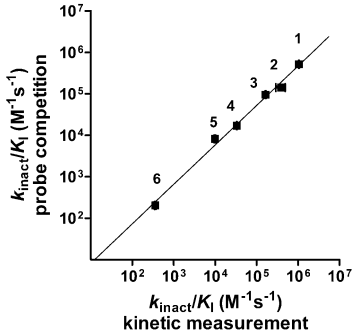

Figure 3. Correlation between k_{inact}/K_I values obtained by conventional kinetic assays and probe-competition assays. Data are given as the mean ± SEM ($n=3$). **1** = CI-1033, **2** = dacomitinib, **3** = WZ8040, **4** = WZ4002, **5** = JAK3 inhibitor V, **6** = Ro 106-9920.

Table 1, the probe-competition assay produced k_{inact}/K_I values that were consistently one half of those obtained by a full kinetic assay approach. The deviation apparently arises from an underestimation of our probe k_{inact}/K_I. The difficulty in determining k_{inact}/K_I to less than a 2-fold variation is also seen in our experimental results from three distinct assay methods (direct binding, kinetic measurement, and probe competition).

To explore the generality of our approach, we derived equations for additional irreversible kinetic models of test compounds and probes. All models produced Equation (1), although the microscopic rate constants comprising the macroscopic parameters vary from mechanism to mechanism (see derivations S2–S4 in the Supporting Information). To investigate the validity of the equations derived above, we performed simulations using KinTek Global Kinetic Explorer (KinTek, Austin, TX).[10] We simulated competitive binding between an irreversible probe and irreversible inhibitors with various values of k_{chem} and k_{inact}/K_I (Scheme S1, and Figure S3A–D in the Supporting Information). In every model examined, the simulation results presented excellent correlations between the original input and calculated values, thus supporting the generality of our approach.

Our k_{inact}/K_I evaluation method that makes use of an irreversible probe has the following three advantages over standard methods; rapid investigation, broad dynamic range, and universal application to all target classes. Prior to this work, the lack of a simple "Cheng–Prusoff relationship" for irreversible inhibitors to relate k_{inact}/K_I for a probe and irreversible test compounds required the determination of a k_{inact}/K_I for each test compound by laborious kinetic measurements. Our method requires obtaining the k_{inact}/K_I of an irreversible probe. Next, one can obtain the k_{inact}/K_I of test compounds through a simple endpoint competition assay. In addition, by examining how the observed results deviate from Equation (1), the probe-competition method offers a quick investigation of inhibitor irreversibility. Reversible inhibitors will retard the binding of an irreversible probe but will not displace the binding of an irreversible probe at infinite time. In a standard IC_{50} assay with reversible ligand (substrates or probes), an irreversible inhibitor shows time-dependent inhibition, with its potency increasing as assay time increases. In the probe-competition method, irreversible inhibitors produce IC_{50} values that are independent of reaction time, while reversible inhibitors will produce IC_{50} values that weaken with increasing assay time (Figure S4A, B in the Supporting Information). Thus, to investigate the irreversibility of a test compound by using our approach, only two IC_{50} values at different time points ($5\times$ half-lives and longer) are required and the shift of the two values elucidates the reversibility of the test compound.

Second, the probe-competition method has an advantage in the detection range of inhibition potencies. Importantly, this range can be increased further by changing the probe concentration. In theory, the probe-competition method has a dynamic range of six orders of magnitude for k_{inact}/K_I (see Note S2 in the Supporting Information). If a wider detection range is required, a different probe with different potency will provide a further detection range of test compound k_{inact}/K_I. Most importantly, the probe-competition method does not depend on rapid equilibrium or steady-state assumptions. Therefore, the method is applicable to highly optimized irreversible inhibitors for which these assumptions no longer hold in the late stages of lead optimization.[11]

Finally, so long as a system satisfies the few assumptions listed above, the method can be applied for the evaluation of irreversible inhibitors of all target classes. Such targets include a nuclear exportin or an enzyme without a fluorogenic substrate for kinetic measurement, for which sensitive and continuous assays are difficult to develop.[12] A key factor of our method is the preparation of an appropriate probe, and in this regard our strategy has an advantage. Unlike reversible binding probes, which require potent affinity for robust assay, irreversible probes need not be highly optimized against their targets since covalent bond formation will always capture the target given sufficient incubation time. Furthermore, the covalent bonding of the probe permits the use of harsh wash conditions, which are unsuitable for reversible probes.

The sensitivity of this approach is governed by a number of factors, including the probe concentration, the protein fraction the filter captures, the specific activity of the probe, and the fractional completion of the assay.[13] The method lacks the signal amplification afforded by following the catalytic activity of the target (should the target be catalytic). Instead, sensitivity is achieved through the use of radiochemistry. If an investigation requires lower protein concentrations, one can offset the corresponding reduction in signal by boosting the specific activity of the probe.

In conclusion, the probe-competition method enables a rapid and quantitative determination of irreversible inhibitor potencies through an endpoint binding assay without

kinetic measurement. This method is simple, high-throughput compatible, widely applicable, and could accelerate efficient lead discovery in the development of drugs based on irreversible inhibitors.

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