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Editorial

Demystifying Functional Parameters for Irreversible Enzyme Inhibitors

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Molecules that possess the ability to form irreversible covalent bonds with their targets are well established in drug development with various examples clinically approved,[1,2] and are featured as the theme of an ACS cross-journal virtual special issue.[3] The formation of a permanently bound complex results in strong pharmacological responses that arise from a prolonged residence time.[4−6] Another appealing attribute of covalent drugs comes from the ability to transform a weak but highly selective compound that would be ineffective as a reversible drug into a highly efficacious and selective agent (e.g., WZ4002 against mutant EGFR).[7] These avenues, among others, have led to a surge of activity to evaluate and discover diverse covalent reactive groups or "warheads" that react with amino acid side chains (e.g., Cys, Lys, etc.) enabling the design of tailored covalent drugs.[8−10] Additionally, variations made to the compound structures to improve noncovalent interactions enable optimization of potency, selectivity, and other properties.[11,12] Collectively, the capacity for medicinal chemists to carry out adequate and efficient evaluation of irreversible inhibitors is critical to affording the desired target profile parameters.

Unlike more conventional noncovalent compounds (denoted "reversible" here—reversible covalent compounds are not covered in this editorial), where determination of IC_{50} or K_i values is commonplace, irreversible agents are more difficult to properly compare in head-to-head assays.[12,13] The main distinguishing attribute of irreversible inhibitors is the time-dependent formation of the covalent bond that can vary significantly between compounds. Therefore, detailed kinetics measurements are required to properly gauge the differences in activity and selectivity of covalent inhibitors.[12,14−16] In this respect, medicinal chemists conduct structure−kinetic relationships (SKRs) for covalent inhibitors, which are akin to the typical structure−activity relationships (SARs).[13] However, due to the elevated complexities and time-demanding nature in characterizing irreversible inhibitors, these assay platforms are not established in many laboratories, and too often the results from these measurements are inappropriately represented.

Our research groups are active in studies and the discovery of irreversible inhibitors that require a detailed understanding of their time-dependent inhibition.[9,11,12,17−22] Many groups in industry and academic laboratories have reported diverse irreversible inhibitor functional parameters, which has been an important development within the field. However, the description of certain parameters and their presentation in the literature is far too commonly made in error, and the confusions that arise because of these inconsistencies should be addressed.[12] Admittedly, some of this confusion likely arises from the similarity in the terms used to characterize reversible and irreversible inhibitors. The overall purpose of this editorial is to provide a resource for both new and established investigators to appreciate and consistently reference the various covalent inhibitor functional parameters that are essential for interpreting irreversible drug activity.

A summary of mechanisms and functional terms is supplied in Figure 1 as a visual reference for understanding the various terms and expressions seen in the characterization of reversible (noncovalent) and irreversible enzyme inhibitors. Key details and learning points are described hereafter:

Noncovalent Reversible Enzyme Inhibitors:

- A reversible inhibitor (I) binds the free enzyme (E) to form the enzyme−inhibitor binary complex (E·I). The reversible inhibitor binds in an equilibrium process governed by the association (k_{on}) and dissociation (k_{off}) rate constants. (Note that these rate constants are often indicated by various alphanumeric subscripts.)
- The binding strength of a reversible inhibitor is commonly quantified by its inhibition constant (K_i), obtained from enzyme activity measurements. This constant is equivalent to the dissociation constant (K_d), which is usually measured through binding experiments. K_i is defined according to the law of mass action as the ratio of the product of the concentrations of free E and I divided by the concentration of E·I. Alternatively, K_i can be expressed as k_{off} divided by k_{on}. The use of the lowercase "i" in the subscript differentiates this parameter from the inactivation constant (K_I) for irreversible inhibitors (see below), which is denoted with an uppercase "I".
- The uppercase "K" denotes an equilibrium constant while lowercase "k" indicates a rate constant, and their italicization implies that these values are unknown variables that are changeable (upper right inset in Figure 1).
- Drug-target residence time (τ), defined as the reciprocal of k_{off}, is the average time the inhibitor remains bound to

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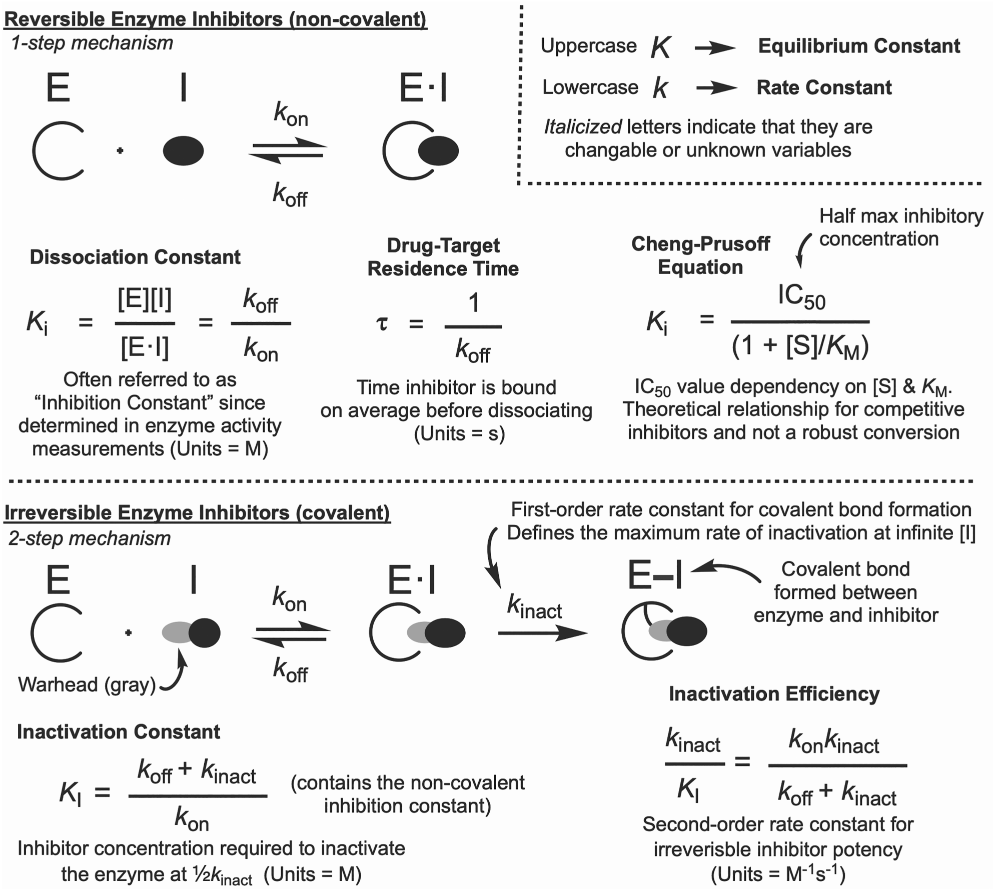

Figure 1. A one-slide summary of the mechanisms of reversible (1-step, noncovalent) and irreversible (2-step, covalent) enzyme inhibitors and accompanying functional parameters.

the enzyme.[4−6] Residence time is a useful predictor of the strength of the pharmacological or biological response of a given inhibitor.[4−6,23] While residence time defines the time a molecule remains bound to the target on average, an often more informative and related metric is the dissociative half-life ($t_{1/2}{}^{diss} = 0.693/k_{off}$) that indicates the length of time required for half of the E·I complex to dissociate to free E and I.

- It is commonly more convenient to determine the half maximal inhibitory concentration (IC_{50}), instead of K_i; however, IC_{50} values suffer limitations such as the tight binding limit and other factors that lead to variabilities.[24] The Cheng−Prusoff formalism accounts for the variability of IC_{50} values with respect to the degree of saturation of the enzyme by the competing substrate, which is governed by the ratio of the substrate (S) concentration to its Michaelis constant (K_M).[25] Moreover, caution should be taken when converting IC_{50} to K_i values by this equation, the discussion of which goes beyond the scope of this editorial.[13,25,26]

Irreversible (Covalent) Enzyme Inhibitors:

- Irreversible enzyme inhibitors typically operate through a two-step mechanism that involves initial reversible binding of the inhibitor to form E·I, governed by k_{on} and k_{off}, followed by the formation of the covalent complex (E−I), described by the first-order rate constant k_{inact}.
- k_{inact} describes the "intramolecular" chemical reaction of E·I to form E−I, and under saturating inhibitor concentrations it is the maximal observed rate of inactivation.
- The inactivation constant (K_I) is defined as the concentration of inhibitor that yields an observed rate constant of inactivation of ${}^{1}/_{2}k_{inact}$.
- Differences in the reversible binding of the initial noncovalent step (i.e., the first in the 2-step mechanism) of the irreversible inhibitor can be qualitatively assessed through comparison of K_I values, although one should apply caution as this term contains k_{inact}.
- K_I and K_i are not interchangeable in much the same way that K_M and K_d for the substrate are not interchangeable. The main distinction between K_I and K_i is that the former includes the contribution of k_{inact}.[13] Strictly, K_I can approximate K_i only when k_{off} is much larger than k_{inact}, which is often the case.
- Overall covalent inhibitor potency is captured by the second-order inactivation efficiency rate constant (k_{inact}/K_I) that is expressed in units of M^{-1} s^{-1}. k_{inact}/K_I is the essential measurement used in medicinal chemistry when assembling SKRs.
- The kinetic parameters governing irreversible inhibitors are mathematically analogous to those in Michaelis−Menten enzyme kinetics. Specifically, k_{inact}, K_I, and k_{inact}/K_I obtained by fitting of inhibitory rate constants versus [I] are mathematically analogous to k_{cat}, K_M, and k_{cat}/K_M, respectively.

- Various protocols have been established for determining k_{inact}, K_I, and k_{inact}/K_I values.[12,14−16] Practically, the majority of these protocols obtain "apparent" values, which are subject to variabilities caused by substrate concentrations and K_M values, and can be readily converted into "true" values to afford more direct comparison of irreversible inhibitor values.[12,14,27]

Recent reports have presented irreversible covalent inhibitor kinetic parameters with a variety of inconsistencies from the correct terms summarized above (Figure 1). In most examples, "k_{inact}/K_I" has been inappropriately presented as "k_{inact}/K_i", which is an apparent confusion over the use of the dissociation constant (K_i, reflecting only the initial reversible binding step) with the inactivation constant (K_I, including binding and covalent bond formation). Additionally, other cases of confusion have arisen where K_i is inappropriately equated to K_I being derived from IC_{50} values. It should be noted that there are instances of authors authentically reporting k_{inact}/K_i values, which are relatively rare.[27,28] Other unclear examples have presented "k_{inact}" as "K_{inact}", where the lowercase and uppercase differences confuse the term being either a rate or equilibrium constant, respectively. In our experience, such discrepancies can be resolved by evaluating their context with the necessary background knowledge on the methods that have been used to derive these parameters.[12,14]

A reasonable and candid opinion of these confusions or misrepresentations of functional parameters may very well be to leave well enough alone since the values obtained are not erroneous or deliberately misleading. Indeed, no intentional harm is done; however, we should take the opportunities to hold ourselves to higher standards in the interest of the field. Where these discrepancies frequently reveal themselves is in conversations with newcomers to medicinal chemistry, namely students and postdocs with training in other fields. For anyone making a sincere effort to afford a complete understanding from the literature, these errors result in intellectual insecurities and propagate inaccuracies into presentations, paper drafts, and other reports that have become increasingly challenging to rectify effectively. Additionally, the conceptional understanding underlying the differences between K_i and K_I can be counteracted if the terms defined in the literature are persistently inconsistent. In essence, *the details matter significantly* for promoting education in our discipline and providing the inspiration for designing improved medicines to treat diverse human diseases. We humbly request that authors, editors, reviewers, and students scrutinize the specifics and definitions/derivations of these various inhibitor parameters to build strong conceptional and mathematical understandings of the nature behind both reversible and irreversible pharmaceutical agents.

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Notes

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After this paper was published ASAP August 8, 2024, Figure 1 was corrected. The revised version was reposted August 16, 2024.