

Mechanism-Based Inhibition: Deriving K_i and k_{inact} Directly from Time-Dependent IC_{50} Values

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The potential of enzyme inhibition of a drug is frequently quantified in terms of IC_{50} values. Although this is a suitable quantity for reversible inhibitors, concerns arise when dealing with irreversible or mechanism-based inhibitors (MBIs). IC_{50} values of MBIs are time dependent, causing serious problems when aiming at ranking different compounds with respect to their inhibitory potential. As a consequence, most studies and ranking schemes related to MBIs rely on the inhibition constant (K_i) and the rate of enzyme inactivation (k_{inact}) rather than on IC_{50} values. In this article, the authors derive a novel relation between potentially time-dependent IC_{50} values and K_i , k_{inact} parameters for different types of inhibition. This allows for direct estimation of K_i and k_{inact} values from time-dependent IC_{50} values, even without the need of additional preincubation experiments. The application of this approach is illustrated using a fluorimetric assay to access the drug-drug interaction potential associated with new chemical entities. The approach can easily be implemented using standard software tools (e.g., XLfit) and may also be suitable for applications where mechanism-based inhibition is a desired mode of action (e.g., at particular pharmacological drug targets). (*Journal of Biomolecular Screening* 2009:913-923)

Key words: irreversible inhibition, time-dependent IC_{50} values, Cheng-Prusoff relation

INTRODUCTION

CHARACTERIZING THE POTENTIAL OF IRREVERSIBLE INACTIVATION of an enzyme by a drug is useful—for example, when aiming at avoiding undesirable inhibition of metabolizing enzymes in the body (e.g., cytochrome P450 enzyme inhibition) or when aiming at efficiently inhibiting target enzymes (e.g., in cancer therapy). The potential of enzyme inhibition is frequently quantified in terms of IC_{50} values, characterizing the degree to which the enzyme activity is inhibited, which are then used, for example, for ranking of new chemical entities in terms of their inhibitory potency.

Inhibition of cytochrome P450 enzymes is a principle mechanism for drug-drug interactions, which may cause severe complications in the clinics.¹ It is the underlying mechanism of some of the most notable drug-drug interactions of greatest magnitude²—for example, paroxetine and CYP2D6,³

clarithromycin and CYP3A,⁴ and furanocoumarins and CYP3A.⁵ To access the drug-drug interaction risks associated with new chemical entities (NCEs), in vitro studies—particularly those using liver microsomes, hepatocytes, or recombinant CYPs—are being applied.^{2,6,7} For a thorough discussion of the different approaches, see Bachmann,⁷ Tucker et al.,⁸ and Zlokarnik et al.⁹ One of the assays being employed is a microplate assay that uses cDNA-expressed human hepatic CYPs and fluorimetric substrates.¹⁰ This assay allows a quick estimation of IC_{50} values, which then can be used to rank compounds according to their drug-drug interaction (DDI) potential.^{11,12} However, to predict the risk of a particular NCE for drug-drug interactions in the clinics, assays based on liver microsomes and drug substrates are much preferred—especially in the case of CYP 3A4, for which multiple binding sites have been described.¹³

Mechanism-based inhibition can also be a desirable property—for example, when targeting enzymes in cancer therapy,¹⁴ inhibiting prostatic acid phosphatases,¹⁵ or zinc proteases.¹⁶

The existing types of enzyme inhibition can be roughly divided into reversible and irreversible inhibition. Although much research has been conducted to understand reversible interactions, much less is known for irreversible or mechanism-based inhibition.^{2,17,18} Although simple and fast approaches based on the fluorimetric assay to identify mechanism-based inhibitors have been described in the literature,^{19,20} the determination of the kinetic parameters of inactivation has been left to

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more complex study designs employing different preincubation schemes.²¹ In the case of reversible inhibition, conversions from IC_{50} into the K_i value are usually performed using the Cheng-Prusoff equations.¹⁹ For mechanism-based inhibition assays, in addition to the K_i value, a second parameter, k_{inact} , accounting for the irreversible inhibition has to be taken into account. This parameter is related to the time-dependent decrease of IC_{50} values for irreversible inhibition.²² In this situation, the Cheng-Prusoff equation results in erroneous predictions.

The objective of this article is to estimate the kinetic parameters K_i and k_{inact} directly from time-dependent IC_{50} measurements without the need to perform additional preincubation experiments. To this end, we develop a general relation between IC_{50} , K_i , and k_{inact} for mechanism-based inhibitors. It is based on a mathematical model of the experimental setup for the determination of the IC_{50} . The assumptions, under which the general relation is valid, reflect the typical experimental conditions of the in vitro assays. We illustrate this new approach using a fluorimetric assay where time-dependent IC_{50} values can easily be obtained by continuously measuring the plate in the fluorescence reader. Results are given for CYP450 isoenzymes 1A2 and 3A4 (i.e., enzymes with well-defined and complex binding characteristics, respectively). Estimated values for K_i and k_{inact} are generally in good agreement with experimental data from the literature.

MATERIAL AND METHODS

Materials

Baculovirus/insect cell-expressed human CYP1A2 and CYP3A4 microsomes (Supersomes), 3-Cyano-7-Ethoxycoumarin (CEC), 7-Benzoyloxy-trifluoromethylcoumarin (BFC), cofactors, glucose-6-phosphate dehydrogenase, and potassium phosphate buffer were obtained from GENTEST/BD Biosciences (Woburn, MA). Test chemicals and their suppliers were as follows: raloxifene and ethinylestradiol were synthesized in the laboratories of Bayer Schering Pharma AG (Berlin, Germany). All other test compounds were purchased from SIGMA-Aldrich (Munich, Germany).

Fluorimetric enzyme inhibition assay. Incubations were conducted in black 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) based on the method described by GENTEST with slight modifications as described in the summary below. All incubations were performed in either 50 mM (CYP 1A2) or 200 mM (CYP 3A4) potassium phosphate buffer at pH 7.4 containing 8.1 μ M NADP⁺, 0.41 mM MgCl₂, 0.4 mM glucose-6-phosphate, and 0.2 IU/mL glucose-6-phosphate dehydrogenase. Final concentrations of cytochrome P450 enzymes and substrates were 2.5 nM CYP1A2, 5 nM CYP3A4, 5 μ M CEC, and 50 μ M BFC. All test compounds were dissolved in acetonitrile and serially diluted (1:3) to 6 final concentrations ranging from 0.041 to 10 μ M, keeping

the final concentration of acetonitrile to 2% in all incubations. The plate with buffer, test compounds, and cofactors was pre-warmed at 37°C, and the reaction was started by adding pre-warmed (37°C) enzyme/substrate mixture. Fluorescence in each well was measured using a PerkinElmer Victor3 multi-label counter with temperature control set to 37°C. Instead of using endpoint measurements, CYP activity was determined at intervals of 2 min up to 30 min after start of reaction using an excitation/emission wavelength of 410/460 nm and 410/530 nm for CYP1A2 and CYP3A4, respectively. Usually, measurements at very early time points are more difficult to obtain (and therefore often more error prone) due to the experimental setup. Because early measurements are not necessarily required for our proposed approach, measurements were taken starting from 5 min. For analysis fluorescence, data were exported to Excel, and IC_{50} s at various time points were determined by fitting the experimental data to a sigmoidal dose-response model using XLfit (IDBS, Guildford, UK).

Models of enzyme inhibition

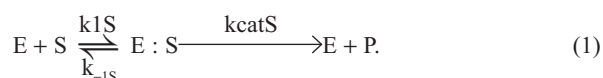
Experimentally, the inhibitory potential of an enzyme inhibitor is determined by measuring the decrease in metabolite formation by the enzyme in the presence of the inhibitor—measured using fluorimetric or liquid chromatography/mass spectrometry (LC/MC) assays.

The IC_{50} value is typically defined as concentration of the inhibitor, at which the velocity of product formation is half the velocity without inhibitor.¹⁹ For reversible inhibition, this is identical to the inhibitor concentration that decreases the metabolite concentration by 50%.

In the sequel, we present a theoretical approach that allows for determining the IC_{50} in a way that exactly matches the experimental setup of the in vitro assay and defines the IC_{50} in terms of the model-theoretical analog of the fluorescent product concentration. As a special case, we regain the well-known Cheng-Prusoff equations, if we impose additional assumptions. These additional assumptions are typically satisfied for reversible inhibition; however, they are clearly violated for the important class of mechanism-based inhibitors.

In the following, we have chosen the 2 examples of competitive inhibition and mechanism-based inhibition to derive a mathematical description of time-dependent CYP inhibition. This allows for illustrating the common parts as well as the differences between our approach and the Cheng-Prusoff approach.

Product formation without inhibition. In the absence of any inhibitor, the product formation by a catalytic enzyme reaction typically comprises the following reaction steps:

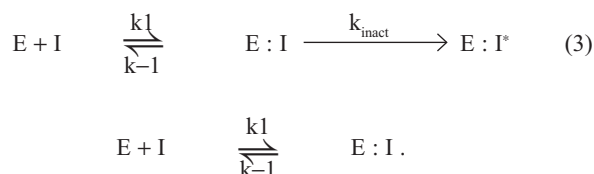


The velocity of product formation is approximated by Michaelis-Menten kinetics:

$$V_0(t) = \frac{d}{dt}P_0(t) = \frac{V_{\max} \cdot S}{K_M + S}, \quad (2)$$

where $V_{\max} = k_{\text{cat}} \cdot E_{\text{cat}}$ denotes the maximal velocity of product formation defined in terms of the functional catalytic enzyme concentration E_{cat} (here identical to the total enzyme concentration), and K_M denotes the substrate concentration, at which $V_0 = V_{\max}/2$.

Competitive inhibition. In the case of competitive inhibition, the inhibitor may form a complex with the free enzyme and thus decrease the rate at which the product is catalyzed. This is shown in the following reaction scheme:



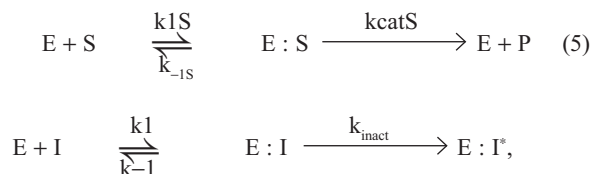
The resulting velocity of product formation is then given by

$$V_I(t) = \frac{d}{dt}P_I(t) = \frac{V_{\max} \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S}, \quad (4)$$

where in addition to equation (2), the parameter $K_i = k_{-1}/k_1$ denotes the dissociation constant of the inhibitor-enzyme complex.

We remark that for the above 2 models, the maximal velocity $V_{\max}$ does not change over time because the underlying functional catalytic enzyme concentration is constant in time. This condition, however, is violated for the following type of inhibition.

Mechanism-based inhibition. This form of inhibition involves, in addition to the competitive inhibition, a reaction that irreversibly transforms the inhibitor-enzyme complex into an “inactive” form, denoted by $E:I^*$. The corresponding reaction scheme is given by



where k_{inact} describes the rate at which the inhibitor-enzyme complex is irreversibly transformed into $E:I^*$. The resulting equation for product formation is given by

$$V_I(t) = \frac{d}{dt}P_I(t) = \frac{V_{\max}(t) \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S}, \quad (6)$$

where, due to the irreversible formation of $E:I^*$, the functional catalytic enzyme concentration $E_{\text{cat}}(t)$ does decrease in time, resulting in a time-dependent maximal velocity $V_{\max}(t) = k_{\text{cat}} \cdot E_{\text{cat}}(t)$. The maximal rate of inactivation is defined by the product of k_{inact} and the active enzyme concentration. The parameter K_i is the inhibitor concentration that results in half the maximal rate.⁴ A detailed description of the relation between K_i and K_i can be found in Mayhew et al.⁴

The resulting time-dependent decrease in functional catalytic enzyme requires establishing an additional evolution equation for $E_{\text{cat}}(t)$. To do so, we note that $E_{\text{tot}} = E_{\text{cat}} + E:I^*(t)$, where E_{tot} denotes the total enzyme concentration in the system (that is assumed to be constant). Under steady-state conditions, the concentration of the enzyme-inhibitor complex $E:I(t)$ over time satisfies

$$E : I(t) = \frac{I}{K_i \left(1 + \frac{S}{K_M}\right) + I} \cdot E_{\text{cat}}(t), \quad (7)$$

from which we may derive the required equation: because $E_{\text{cat}}(t) = E_{\text{tot}} - E:I^*(t)$ and $d/dt E_{\text{tot}} = 0$, it is $\frac{d}{dt}E : I^*(t) = k_{\text{inact}} \cdot E : I(t)$, which, combined with equation (7), results in

$$\frac{d}{dt}E_{\text{cat}}(t) = -k_{\text{inact}} \cdot \eta_I \cdot E_{\text{cat}}, \quad (8)$$

with initial condition $E_{\text{cat}}(0) = E_{\text{tot}}$ and

$$\eta_I = \frac{I}{K_i \left(1 + \frac{S}{K_M}\right) + I}. \quad (9)$$

To highlight the dependence of $V_{\max}(t)$ on E_{cat} in equation (6), we rewrite (6) as

$$\frac{d}{dt}P_I(t) = \frac{k_{\text{cat}} \cdot E_{\text{cat}}(t) \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S}. \quad (10)$$

Control and the definition of IC_{50} . In many experimental approaches to determine IC_{50} values, the increase in fluorescent product concentration is measured. Unlike many other theoretical approaches, our definition of the IC_{50} directly builds on the fluorescent product concentration $P(t)$ at time t . Let us define the control, a function of time t and inhibitor concentration I , as

$$\text{control}(t, I) = \frac{P_I(t)}{P_0(t)}, \quad (11)$$

where $P_I(t)$ and $P_0(t)$ denote the concentration of product at time t in the presence of an inhibitor (at concentration I),

respectively, in the absence of any inhibitor. Based on the control, the IC_{50} value is then defined as the inhibitor concentration at which the product concentration is half the concentration of the system without inhibitor, hence

$$\text{control}(t, IC_{50}) = \frac{1}{2} \Rightarrow P_{IC_{50}}(t) = \frac{1}{2} P_0(t). \quad (12)$$

This characterization is used to determine the relation between IC_{50} , K_i , and k_{inact} . The IC_{50} may depend on time, as is the case for mechanism-based inhibition. We note that the general solution to equations (2) and (4) is given by

$$P_I(t) = P(0) + \int_0^t V_I(\tau) d\tau \quad (13)$$

with possibly time-varying velocities of product formation $V_I(t)$ and $V_0(t)$ in the presence and absence of the inhibitor, respectively. Under the *addition assumptions* that $V_0(t)$ and $V_I(t)$ are constant in time, and $P_0(0) = P_I(0) = 0$, equation (11) simplifies to

$$\text{control}(t, I) = \frac{V_I(t)}{V_0(t)} \Rightarrow V_{IC_{50}} = \frac{1}{2} V_0, \quad (14)$$

which is the starting point for the derivation of the well-known Cheng-Prusoff relation between IC_{50} and K_i . These additional assumptions ($V_0(t)$ and $V_I(t)$ are constant in time and $P_0(0) = P_I(0) = 0$) are satisfied for reversible inhibitors (see Cheng and Prusoff¹⁹ for other types of reversible inhibitors), thereby including competitive inhibitors; however, they are not satisfied for mechanism-based inhibitors, as can be seen from equation (6). This clearly shows that the Cheng-Prusoff approach must necessarily fail when aiming at mechanism-based inhibition.

Cheng-Prusoff equation for competitive inhibition

Usually, the in vitro assay is set up in such a way that (A1) the amount of substrate does not change significantly during the performance of the assay (i.e., realized by choosing $S \gg E_{tot}$), (A2) the amount of inhibitor does not change significantly in time, and (A3) the amount of catalytically active enzyme is constant in time. Under these assumptions, the resulting product formation velocities in equations (2) and (1) are constant in time. Hence, assuming zero initial product concentration—that is, $P_0(0) = P_I(0) = 0$ (which is realized by subtracting background fluorescence)—we obtain

$$P_0(t) = \frac{V_{max} \cdot S}{K_M + S} \cdot t \quad \text{and} \quad P_I(t) = \frac{V_{max} \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S} \cdot t. \quad (15)$$

Finally, the IC_{50} is determined by

$$\frac{1}{2} = \frac{P_{IC_{50}}(t)}{P_0(t)} = \frac{K_M + S}{K_M \left(1 + \frac{IC_{50}}{K_i}\right) + S}, \quad (16)$$

which yields the well-known Cheng-Prusoff equation for competitive inhibition¹⁹:

$$K_i = \frac{K_M}{K_M + S} \cdot IC_{50}. \quad (17)$$

This shows the consistency of our approach to the well-established Cheng-Prusoff equation for competitive inhibition. Equations for other types of reversible inhibition can be derived in the same manner; for details, see Cheng and Prusoff.¹⁹

Central relation between time-dependent $IC_{50}(t)$ values and the parameters K_i and k_{inact}

For mechanism-based inhibition, we assume that (A1) the amount of substrate does not change significantly during the performance of the assay, and (A2) the amount of inhibitor does not change significantly in time. We do not assume that the amount of catalytically active enzyme is constant in time. Under these assumptions, we may explicitly solve for the fate of the catalytically active enzyme E_{cat} in equation (8) because under these 2 assumptions, η_I in (9) is constant in time:

$$E_{cat}(t) = E_{cat}(0) \cdot e^{-k_{inact} \cdot \eta_I \cdot t}. \quad (18)$$

Inserting $E_{cat}(t)$ into equation (10) results in

$$\frac{d}{dt} P_I(t) = \frac{k_{cat} \cdot E_{cat}(0) \cdot e^{-k_{inact} \cdot \eta_I \cdot t} \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S}. \quad (19)$$

Solving (19) with $P_I(0) = 0$ yields

$$\begin{aligned} P_I(t) &= \frac{V_{max} \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S} \int_0^t e^{-k_{inact} \cdot \eta_I \cdot \tau} d\tau \\ &= \frac{V_{max} \cdot S}{K_M \left(1 + \frac{I}{K_i}\right) + S} \left(\frac{1 - e^{-k_{inact} \cdot \eta_I \cdot t}}{k_{inact} \cdot \eta_I} \right) \end{aligned} \quad (20)$$

In order to determine the $IC_{50}(t)$, we compute

$$P_{IC_{50}}(t) = \frac{V_{max} \cdot S}{K_M \left(1 + \frac{IC_{50}(t)}{K_i}\right) + S} \left(\frac{1 - e^{-k_{inact} \cdot \eta_{IC_{50}} \cdot t}}{k_{inact} \cdot \eta_{IC_{50}}} \right), \quad (21)$$

with $\eta_{IC_{50}}$ defined by (9) with $I = IC_{50}$. Exploiting equation (12), we obtain

$$\frac{1}{2} = \frac{P_{IC_{50}}(t)}{P_0(t)} = \frac{K_M + S}{K_M \left(1 + \frac{IC_{50}}{K_I}\right) + S} \cdot \frac{1}{t} \cdot \frac{1 - e^{-k_{inact} \cdot \eta_{IC_{50}} \cdot t}}{k_{inact} \cdot \eta_{IC_{50}}} \quad (22)$$

This finally yields the central relation between time-dependent $IC_{50}(t)$ values and the parameters K_I and k_{inact} .

$$IC_{50}(t) = K_I \left(1 + \frac{S}{K_M}\right) \cdot \frac{2 - 2e^{-\eta_{IC_{50}} \cdot k_{inact} \cdot t}}{\eta_{IC_{50}} \cdot k_{inact} \cdot t} - 1 \quad \text{with} \quad \eta_{IC_{50}} = \frac{IC_{50}(t)}{K_I \left(1 + \frac{S}{K_M}\right) + IC_{50}(t)} \quad (23)$$

The above equation illustrates that there is a specific curve of IC_{50} values over time for every combination of K_I and k_{inact} (see **Fig. 1**). In a typical experimental setup, the parameters S , K_M , and I , as well as a series of measured time-dependent IC_{50} values $IC_{50}(1), \dots, IC_{50}(n)$ at different points in time $t_1, \dots, t_n$, are known. The task is then to determine K_I and k_{inact} , which can be done by a least squares fit using equation (23)—for example, by adding the above equation as a new model in XLfit and computing K_I and k_{inact} by fitting the right side of the equation to the measured IC_{50} values. To compute the right-hand side, the input parameter $\eta_{IC_{50}}$ is determined based on the measured IC_{50} values. As a result, the measured IC_{50} values enter twice in the process of estimating K_I and k_{inact} : as an input to the right-hand side (via $\eta_{IC_{50}}$) and as experimental data to fit the prediction of the model in equation (23). As is well known, the precision of the estimated parameters typically increases with the number of experimental data points (IC_{50} values) available. Therefore, using the proposed fluorimetric assay, the precision of K_I and k_{inact} estimation can be increased by increasing the frequency of fluorescence readouts. **Figure 2** shows the complete process of determination of K_I and k_{inact} . A step-by-step tutorial for realization in XLfit can be found online in the Supplement to this article at <http://jbx.sagepub.com/supplemental>.

Solving equation (23) for K_I , we nicely see the analogy to the original Cheng-Prusoff equation:

$$K_I = \left(\frac{K_M}{K_M + S}\right) \cdot IC_{50}(t) \cdot C(k_{inact}, t, \eta_{IC_{50}}), \quad (24)$$

where the factor C depends on k_{inact} , time, and $\eta_{IC_{50}}$:

$$C(k_{inact}, t, \eta_{IC_{50}}) = \frac{2 - 2e^{-\eta_{IC_{50}} \cdot k_{inact} \cdot t}}{\eta_{IC_{50}} \cdot k_{inact} \cdot t} - 1 \quad (25)$$

Hence the central relation (23) can be interpreted as a corrected Cheng-Prusoff equation accounting for the

time-dependent loss of the functional catalytic enzyme. In the limit of vanishing k_{inact} , we obtain $C = 1$ using l'Hopital's rule, which is consistent with equation (17). In previous attempts, this factor has never been accounted for correctly (e.g., Maurer et al.²³).

RESULTS

The derived central relation (23) between K_I , k_{inact} , and the IC_{50} values was validated *in silico* by generating concentration-time profiles of the resulting product over 30 min using equation (19) (see **Fig. 3**, left). Based on these data, theoretical IC_{50} values were calculated at each minute based on equation (12) and used to validate relation (23). In addition, we show the prediction based on the relation (B3) derived in Maurer et al.²³ As shown in **Figure 3** (right), the herein derived relation is in excellent agreement with the theoretical IC_{50} values. In contrast, the relationship derived by Maurer et al. differs substantially from the IC_{50} values; in particular, it predicts negative IC_{50} values.

The performance of the proposed approach was further evaluated based on fluorimetric assay data of 7 well-known mechanism-based inhibitors (MBIs) for CYP450 isoforms 1A2 and 3A4. All MBIs showed the time-dependent decrease in IC_{50} values (see **Fig. 4**). Clearly, the model predictions nicely describe the decrease of IC_{50} values due to mechanism-based inhibition for all 6 inhibitors (see **Fig. 5**).

To access the accuracy of the estimated parameters of inhibition, we provide a comparison to literature values that were obtained in human liver microsomes by use of the Kitz-Wilson or nonlinear regression method. Based on the known parameters S and K_M , the inhibition parameters K_I and k_{inact} have been estimated by fitting model (23) to the time-dependent IC_{50} data. In **Table 1**, the estimated values of K_I and k_{inact} (based on single fluorimetric assay experiments) are compared to values of K_I and k_{inact} estimated by classical data analysis methods (based on a series of preincubation experiments). As can be inferred from **Table 1**, generally there is a good agreement between our estimates and K_I and k_{inact} values based on classical data analysis methods. Calculated k_{inact} values were within a maximal 2.5-fold error range of a literature value. K_I values were also within a 2.5-fold error range, except for ethinylestradiol and raloxifene.

Repeated experiments for verapamil, furafylline, and ethinylestradiol with different sampling protocols were used to access the impact of experimental variability. Model predictions are in good agreement with experimental IC_{50} values even for small numbers of sampling points (**Fig. 5**). The resulting estimates of K_I and k_{inact} for different numbers of sampling points can be found in **Table 1**.

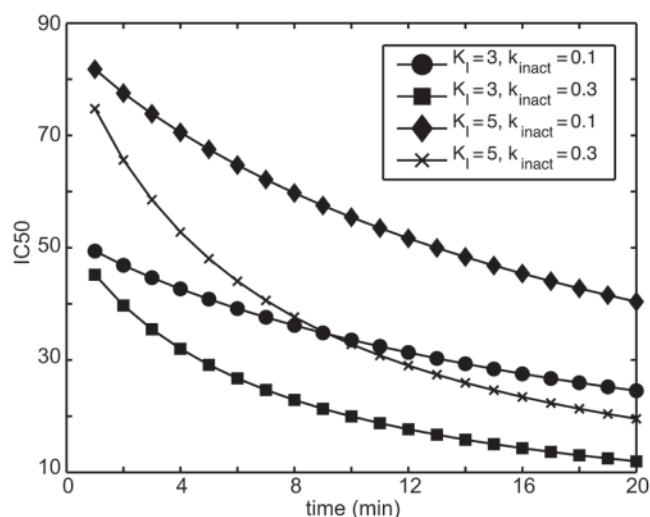


FIG. 1. Illustration of the influence of different combinations of K_I and k_{inact} on the time dependence of the IC_{50} . Given a series of IC_{50} values over time, this in turn allows us to determine the K_I and k_{inact} values by a least squares fit using equation (23).

DISCUSSION

Irreversible inactivation of enzymes by an inhibitor can be undesirable when drug-metabolizing enzymes are inhibited (e.g., cytochrome P450 enzymes) or beneficial when drug target enzymes are efficiently inhibited. In both cases, knowledge of the parameters of inhibition K_I and k_{inact} is useful to characterize the inhibitory potential. In this article, we derive a novel relation to directly estimate these 2 parameters from time-dependent IC_{50} values without the need of a series of measurements with increasing preincubation time. The proposed approach is generally applicable to experimental assays that provide a series of time-dependent IC_{50} values.

The theoretical derivation is tightly coupled to the experimental conduct of the assay—that is, the derivation is based only on assumptions, under which the *in vitro* experiment is typically performed. Under these assumptions, equation (23) provides a mathematically founded relation between the time dependence of IC_{50} values and the parameters of inhibition K_I and k_{inact} . To the best of our knowledge, it is the first time that this key relation has been reported. Previous attempts were based on relating the IC_{50} value directly to reaction velocities.²³ During the performance of the assays, however, product concentrations rather than reaction velocities are measured. Equation (14) states that considering concentrations or reaction velocities is equivalent for reversible inhibition. However, this is not the case for mechanism-based inhibition. The difference between the 2 approaches is apparent from quality of predictions shown in **Figure 3**. By definition, the velocity of product formation decreases over time

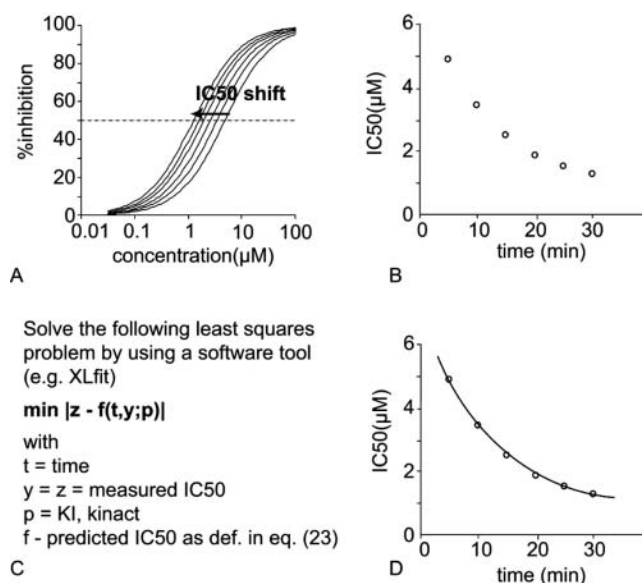


FIG. 2. Principle procedure to obtain K_I and k_{inact} values from time-dependent CYP inhibition measurements. (A) Measurement of inhibition curves at several time points during incubation of test compound with enzyme and substrate. Irreversible inhibitors are characterized by a time-dependent left shift of the curve as indicated by the arrow. (B) Plot of IC_{50} values against incubation time. (C, D) Fitting of the model to the IC_{50} values (e.g., by using XLfit) to obtain estimates of K_I and k_{inact} .

in the presence of irreversible inhibitors. Consequently, comparing reaction velocities at a particular time is only a snapshot of the inhibition process at that time. However, it does not take into account the gradual inactivation process and its accumulated effect up to that time. This gradual decay of the product formation velocity over time, however, has to be considered, which is realized in our approach by integrating the velocity over the incubation time, resulting in the term $\eta_{IC_{50}} \cdot k_{\text{inact}} \cdot t$ in the denominator of the central relation (23). A characterization solely based on reaction velocities is questionable.

To access the drug-drug interaction potential of new compounds, *in vitro* studies using liver microsomes, hepatocytes, or recombinant CYPs are being applied.^{2,7} We applied our approach in the context of drug-drug interaction because experimental data are readily available and because irreversible inactivation is the underlying mechanism of some of the most important drug-drug interactions of greatest magnitude²—for example, paroxetine and CYP2D6,³ clarithromycin and CYP3A,⁴ and furanocoumarins and CYP3A.⁵

As reported by Yan et al.²² and Naritomi et al.,²¹ irreversible CYP inhibition can easily be distinguished from reversible inhibition by comparing the time-dependent pattern of IC_{50} values from CYP inhibition experiments. If a fluorimetric assay is used, data for different time points can easily be obtained by

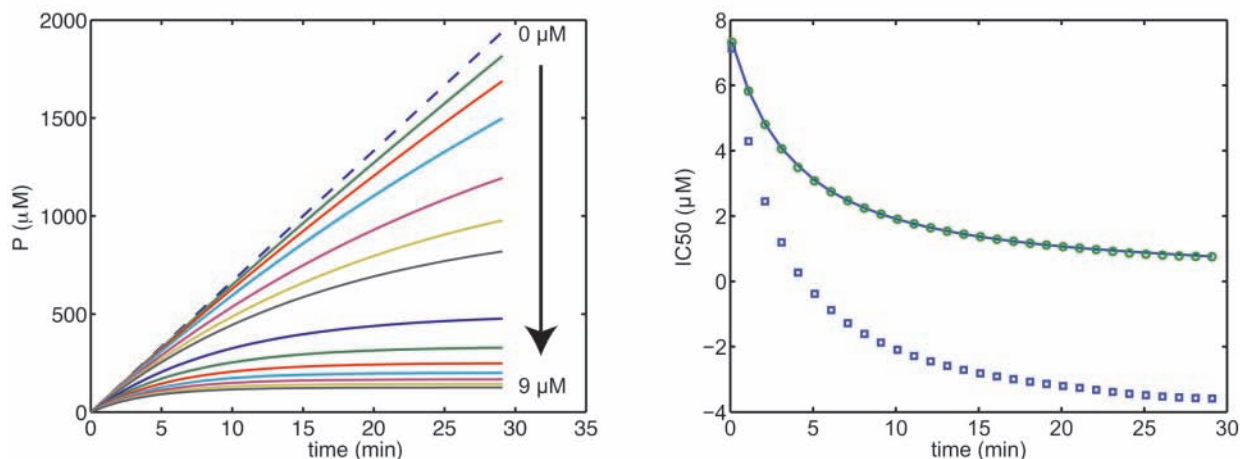


FIG. 3. Validation of equation (23) and comparison with the relation derived in Maurer et al.²³ Left: Using equation (19), product formulation over 30 min was simulated with increasing concentrations of a mechanism-based inhibitor from 0 μM (top) to 9 μM (bottom). The parameters V_{max} , K_m , K_i , S , and k_{inact} were set to 100 $\mu\text{M}/\text{min}$, 2.5 μM , 2.5 μM , 5 μM , and 0.5 min^{-1} , respectively (as in Maurer et al.²³). Right: Comparison of theoretical IC_{50} values (solid line) with predictions resulting from the herein proposed relation equation (23) (circles) and the alternative relation (equation (B3) in Maurer et al.²³) (squares).

reading the fluorescence intensity for an individual plate at several points in time without stopping the enzymatic reactions. Therefore, estimating IC_{50} values over time using microplate assays is a simple and attractive method to detect irreversible inhibitors.²¹ Although this approach allowed for the *detection* of mechanism-based inhibition, the herein proposed application makes extended use of the *same* data to also derive K_i and k_{inact} values, therefore moving from a simple yes/no statement toward a more quantitative assessment of the DDI potential.

Typically, the experimental determination of K_i and k_{inact} values required the performance of additional preincubation experiments,² whose data are subsequently analyzed by use of the Kitz-Wilson or nonlinear regression method.²⁴ The presented method, however, allows for the direct estimation of K_i and k_{inact} from the set of time-dependent IC_{50} values.

We have analyzed the accuracy and reliability of the predictions based on recombinant CYPs and the herein proposed approach for various drugs and the cytochrome P450 isoforms 1A2 and 3A4. The estimated K_i and k_{inact} values compare well to published data, except for the K_i value of ethinylestradiol and raloxifene. This might be due to limitations of the fluorimetric assay, such as interference from fluorescent inhibitors. In addition, several CYP isoforms may contribute to the formation of the ultimate CYP inhibitor. Because the substrates for fluorescent assays are not CYP selective and necessitate the use of recombinant single-enzyme systems, the inhibitory effect of metabolites generated by one CYP on other CYPs cannot be tested.^{25,26} However, despite these problems, time-dependent fluorimetric assays are used to study compounds with respect to potency of MBIs.²⁷⁻²⁹

To evaluate the robustness of our approach to experimental variability, we estimated K_i and k_{inact} based on different sampling protocols and repeated experiments for verapamil, furafylline, and ethinylestradiol. The good agreement between our model predictions and the experimental IC_{50} values shows that the derived relation is capable of predicting time-dependent IC_{50} values as different, as shown in **Figure 5**.

Estimated K_i values varied roughly 2-fold, whereas k_{inact} values varied to a larger extent (almost 10-fold). As expected, this shows the variability of the underlying biological system, which can also be observed in the reported literature values (e.g., furafylline or erythromycin). Given the inherent variability of the literature values, results are in good agreement with published experimental data (i.e., less than 2.5-fold deviation from a literature value).

In summary, the presented approach allows for the direct use of time-dependent IC_{50} data to estimate the kinetic parameters of inactivation (K_i , k_{inact}). Streamlining the process of evaluating mechanism-based enzyme inactivation is of great interest and importance to the drug discovery process. The design of new experimental approaches aiming at mechanism-based inhibitors necessitates the knowledge of the relation between time-dependent IC_{50} values and the underlying parameters of inactivation K_i and k_{inact} (see, e.g., Berry and Zhao³⁰). The herein derived central relation provides the required theoretical foundation. We would like to point out that the presented approach will also be very useful in cases where mechanism-based inhibition is a desired property (e.g., targeting enzymes in cancer therapy).¹⁴

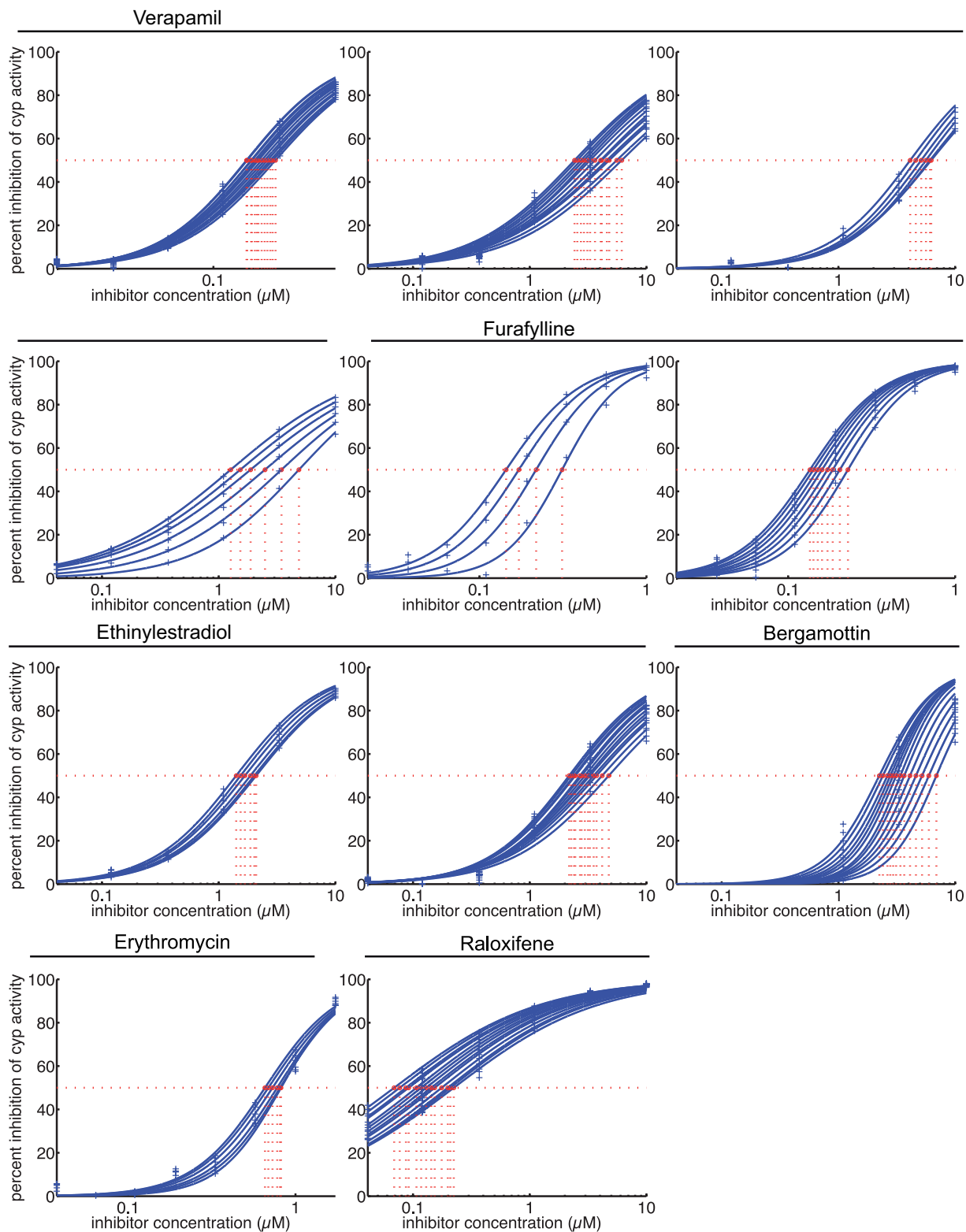


FIG. 4. Measured inhibition curves for the 7 mechanism-based CYP inhibitors (including replicates) with different inhibitor concentrations and at different points in time. IC_{50} values have been obtained for each point in time by fitting a dose-response model with variable Hill parameter to the data (curves). The obtained IC_{50} values have been used subsequently to estimate K_i and k_{inact} values for each inhibitor.

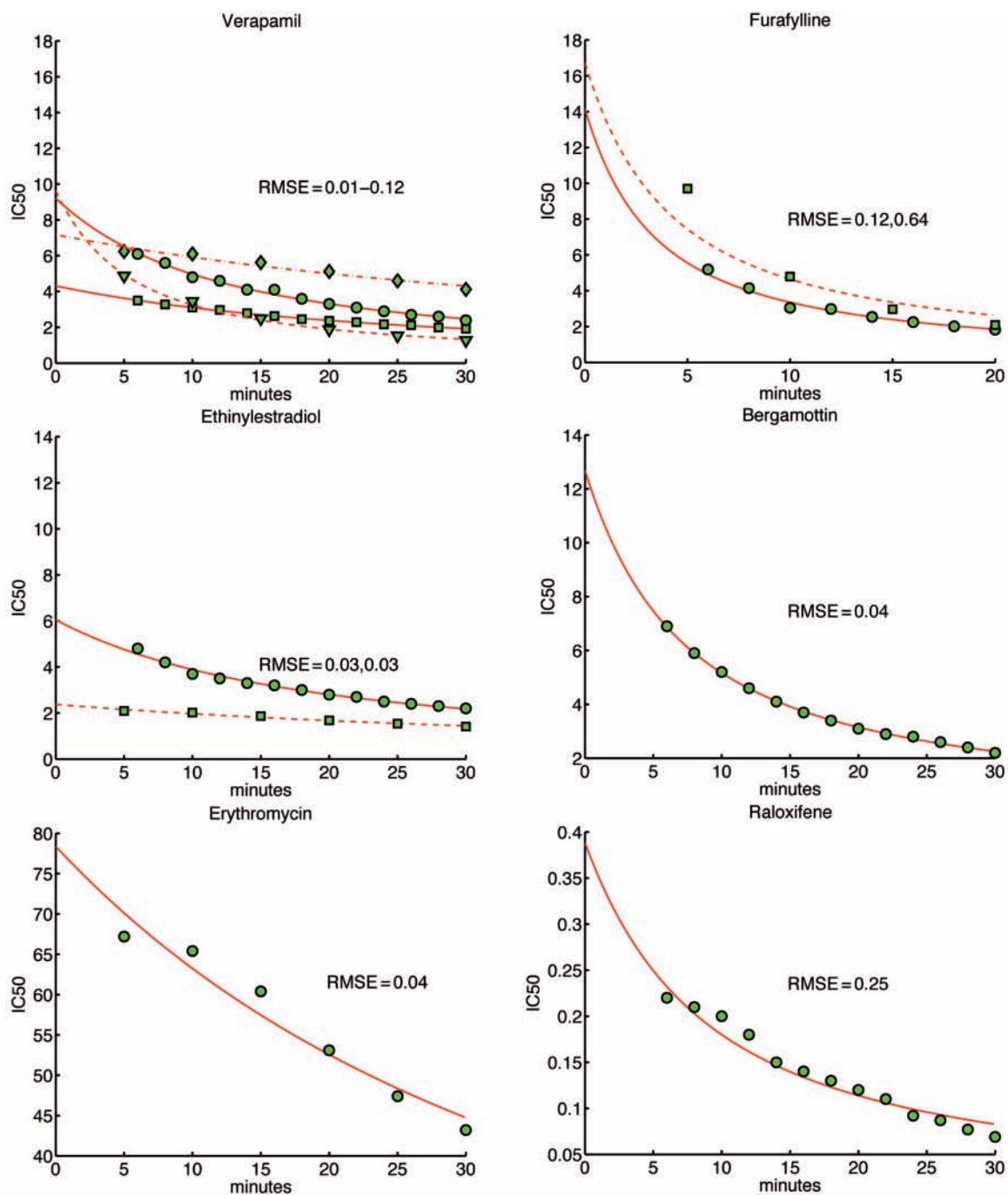


FIG. 5. Illustrative time-dependent IC_{50} values from CYP P450 inhibition experiments for 7 different mechanism-based inhibitors of CYP isoforms 3A4 and 1A2. IC_{50} values have been determined at different points in time. For each set of IC_{50} values, a curve is fitted by using the developed central relation (23), which represents a specific K_i and k_{inact} value with goodness of fit given as a normalized root mean square error (RMSE). The obtained values are listed in **Table 1**. IC_{50} values are given in μM. Each plot is an example of up to 4 individual experiments.

Table 1. Comparison of K_i and k_{inact} Values Calculated by the Herein Proposed Method and Literature Data

Substance	CYP isoform	K_i	K_i fold err.	k_{inact}	k_{inact} fold err.	Ref.	Substrate used in ref.
			< 2.5 > 2.5		< 2.5 > 2.5		
Verapamil	3A4		X		X	Yeo and Yeo, 2001; Polasek et al., 2004 ^{31, 32}	testosterone, paclitaxel
Furafylline	1A2		X		X	Clarke et al., 1994; Kunze and Trager, 1993 ^{33, 34}	warfarin
Erythromycin	3A4		X		X	Yamano et al., 2001; Chan and Delucchi, 2000 ^{35, 36}	midazolam, testosterone
Ethinylestradiol	3A4		X		X	Lin et al., 2002 ³⁷	testosterone
Bergamottin	3A4		X		X	He et al., 1998 ³⁸	testosterone
Raloxifene	3A4		X		X	Chen et al., 2002 ³⁹	testosterone

For each compound, a potential range of data for K_i (here 0-30, or 0-90) and k_{inact} (here 0-1) has been depicted. Values with bars pointing downwards refer to literature data, whereas bars pointing upwards refer to own determinations. Estimates by using the here proposed method are based on experimental data from CYP inhibition assays using fluorescent substrates described earlier. Literature data are based on human liver microsomes. Units: K_i in μM and k_{inact} in min^{-1} .

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