

# **Mechanism-based Inhibition: Deriving KI and kinact directly from Time-Dependent IC50 Values**

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Short title: Time-dependent Enzyme Inhibition

Words: 5712

## Abstract

The potential of enzyme inhibition of a drug is frequently quantified in terms of IC<sub>50</sub> values. While this is a suitable quantity for reversible inhibitors, concerns arise when dealing with irreversible, or mechanism-based inhibitors (MBI). IC<sub>50</sub> values of MBI 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 MBI rely on the inhibition constant ( $K_I$ ) and the rate of enzyme inactivation ( $k_{inact}$ ) rather than on IC<sub>50</sub> values. In this article we derive a novel relation between potentially time-dependent IC<sub>50</sub> 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<sub>50</sub> values, even without the need of additional pre-incubation experiments. The application of this approach is illustrated using a fluorimetric assay to assess 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 actions, e.g., at particular pharmacological drug targets.

**Keywords:** irreversible inhibition, time-dependent IC<sub>50</sub> values, Cheng-Prusoff relation

## Introduction

Characterizing the potential of irreversible inactivation of an enzyme by a drug is useful, e.g., when aiming at avoiding undesirable inhibition of metabolising 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<sub>50</sub> values, characterizing the degree to which the enzyme activity is inhibited, which are then used, e.g., 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 (Kalgutkar et al., 2007). It is the underlying mechanism of some of the most notable drug-drug interactions of greatest magnitude (Venkatakrisnan et al., 2003), e.g. paroxetine and CYP2D6 (Bertelsen et al., 2003), clarithromycin and CYP3A (Mayhew et al., 2000), furanocoumarins and CYP3A (Greenblatt et al., 2003). In order to assess the drug-drug interaction risks associated with new chemical entities (NCE), *in vitro* studies—particularly those utilizing liver microsomes, hepatocytes or recombinant CYPs—are being applied (Di et al., 2007, Bachmann et al., 2006, Venkatakrisnan et al., 2003). For a thorough discussion of the different approaches see Bachmann et al., 2006; Tucker et al, 2001; Zlokarnik et al., 2005. One of the assays being employed is a microtiter-plate assay that uses cDNA expressed human hepatic CYPs and fluorimetric substrates (Crespi et al., 1997). This assay allows a quick estimation of IC<sub>50</sub> values, which then can be used to rank compounds according to their DDI potential (Krippendorff et al., 2007, Turpeinen et al., 2006). However, in order 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 case of CYP 3A4 for which multiple binding sites have been described (Scott and Halpert, 2005).

Mechanism-based inhibition can also be a desirable property, e.g. when targeting enzymes in cancer therapy (Bruno and Njar, 2007), inhibiting prostatic acid phosphatases (Myers and Widlanski 1993) or zinc proteases (Kim and Mobashery 2001).

The existing types of enzyme inhibition can be roughly divided into reversible and irreversible inhibition. While much research has been conducted to understand reversible interactions, much less is known for irreversible or mechanism based inhibition (Obach et al., 2005, 2006, 2007, Venkatakrishnan et al., 2003). Although simple and fast approaches based on the fluorimetric assay to identify mechanism based inhibitors have been described in the literature (Cheng and Prusoff, 1973, He et al., 1998) the determination of the kinetic parameters of inactivation has been left to more complex study designs employing different pre-incubation schemes (Naritomi et al., 2004). In case of reversible inhibition conversions from IC<sub>50</sub> into the K<sub>I</sub> value are usually performed using the Cheng-Prusoff equations (Cheng and Prusoff 1973). For mechanism-based inhibition assays, in addition to the K<sub>I</sub> value, a second parameter  $k_{\text{inact}}$  accounting for the irreversible inhibition has to be taken into account. This parameter is related to the time-dependent decrease of IC<sub>50</sub> values for irreversible inhibition (Yan et al., 2002). In this situation, the Cheng-Prusoff equation results in erroneous predictions.

The objective of this paper is to estimate the kinetic parameters K<sub>I</sub> and  $k_{\text{inact}}$  directly from time-dependent IC<sub>50</sub> measurements without the need to perform additional pre-incubation experiments. To this end, we develop a general relation between IC<sub>50</sub>, K<sub>I</sub> and  $k_{\text{inact}}$  for mechanism-based inhibitors. It is based on a mathematical model of the experimental setup for the determination of the IC<sub>50</sub>. 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<sub>50</sub> values can easily be obtained by continuously measuring the plate in the fluorescence reader. Results are given for CYP450 iso-enzymes 1A2 and 3A4, i.e., enzymes with well-defined and complex binding characteristics, respectively. Estimated values for K<sub>I</sub> and  $k_{\text{inact}}$  are generally in good agreement with experimental data from literature.

# Material and Methods

## 2.1 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: Raloxifen 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.

### 2.1.1 Fluorimetric Enzyme Inhibition Assay

Incubations were conducted in black 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) based on the method described by GENTEST with slight modifications as described in 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  $\mu$ M NADP<sup>+</sup>, 0.41 mM MgCl<sub>2</sub>, 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  $\mu$ M CEC and 50  $\mu$ M BFC. All test compounds were dissolved in acetonitrile and serially diluted (1:3) to six final concentrations ranging from 0.041 to 10  $\mu$ 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 multilable counter with temperature control set to 37 °C. Instead of using end-point measurements CYP activity was determined at intervals of two minutes up to 30 min after start of reaction using 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. Since early measurements are not necessarily required for our proposed approach, measurements were taken starting from 5min. For analysis fluorescence data were exported to Excel and IC50s at various time-points were determined by fitting the experimental data to a sigmoidal dose-response model using *XLfit* (IDBS, Guilford U.K.).

## 2.2 Models of enzyme inhibition

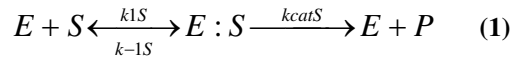
Experimentally, the inhibitory potential of an enzyme inhibitor is determined by measuring the decrease in metabolite formation by the enzyme in the presence of the inhibitor—measured using a fluorimetric or liquid chromatography-mass spectrometry (LC-MC) assays.

The IC50 value is typically defined as concentration of the inhibitor, at which the velocity of product formation is half the velocity without inhibitor (Cheng and Prusoff, 1973). For reversible inhibition, this is identical to the inhibitor concentration that decreases the metabolite concentration by 50%.

In the sequel we will present a theoretical approach that allows for determining the IC50 in a way that exactly matches the experimental setup of the *in vitro* assay and defines the IC50 in terms of the model-theoretical analogue 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 two 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:

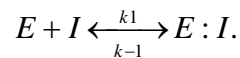
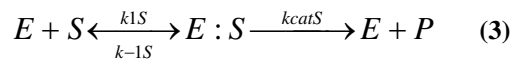


The velocity of product formation is approximated by Michaelis-Menten kinetics:

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

where  $V_{\max} = k_{\text{cat}} S * E_{\text{cat}}$  denotes the maximal velocity of product formation defined in terms of the functional catalytic enzyme concentration  $E_{\text{cat}}$  (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 catalysed. This is shown in the following reaction scheme



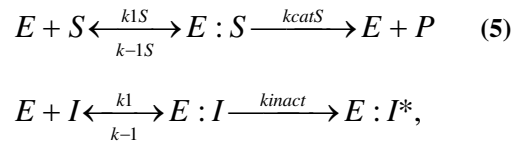
The resulting velocity of product formation is then given by

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

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

We remark that for the above two models, the maximal velocity  $V_{\max}$  does not change over time, since 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 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  $E_{cat}(t)$  does decrease in time, resulting in a time-dependent maximal velocity  $V_{\max}(t) = k_{cat} \cdot E_{cat}(t)$ . 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 (Mayhew et al., 2000). A detailed description of the relation between  $K_i$  and  $K_i$  can be found in (Mayhew et al., 2000).

The resulting time-dependent decrease in functional catalytic enzyme requires establishing an additional evolution equation for  $E_{cat}(t)$ . To do so, we note that  $E_{tot} = E_{cat} + E:I^*(t)$ , 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

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

from which we may derive the required equation: Since  $E_{cat}(t) = E_{tot} - E:I^*(t)$  and  $d/dt E_{tot} = 0$ , it is

$$\frac{d}{dt} E:I^*(t) = k_{inact} \cdot E:I(t), \text{ which combined with Eq. (7) results in}$$

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

with initial condition  $E_{cat}(0) = E_{tot}$  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 Eq. (6), we rewrite (6) as:

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

**Control and the definition of IC50.** In many experimental approaches to determine IC50 values, the increase in fluorescent product *concentration* is measured. Unlike many other theoretical approaches, our definition of the IC50 directly builds upon the fluorescent product concentration  $P(t)$  at time  $t$ . Let us define the control, a function of time  $t$  and inhibitor concentration  $I$ , as

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

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 IC50 value is then defined as the inhibitor concentration at which the product concentration is half the concentration of the system without inhibitor, hence

$$control(t, IC50) = \frac{1}{2} \Rightarrow P_{IC50}(t) = \frac{1}{2} P_0(t). \quad (12)$$

This characterization is used to determine the relation between IC50, KI and  $k_{inact}$ . The IC50 may depend on time, as is the case, e.g., for mechanism-based inhibition. We note that the general solution to Eqs. (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  $P_0(0) = P_I(0) = 0$ , Eq. (11) simplifies to

$$\text{control}(t,I) = \frac{V_I(t)}{V_0(t)} \Rightarrow V_{IC50} = \frac{1}{2}V_0, \quad (14)$$

which is the starting point for the derivation of the well-known Cheng-Prusoff relation between IC50 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 Prusoff, 1973) for other types of reversible inhibitors), thereby including competitive inhibitors; however they are not satisfied for mechanism-based inhibitors, as can be seen from eq. (6). This clearly shows that the Cheng-Prusoff approach must necessarily fail when aiming at mechanism-based inhibition.

### 2.3 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  $S \gg E_{\text{tot}}$ , (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 Eqs. (2) and (1) are constant in time. Hence, assuming zero initial product concentration, i.e.,  $P_0(0)=P_I(0)=0$  (which is realized by subtracting background fluorescence) we obtain

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

Finally, the IC50 is determined by

$$\frac{1}{2} = \frac{P_{IC50}(t)}{P_0(t)} = \frac{K_M + S}{K_M \left(1 + \frac{IC50}{K_i}\right) + S}, \quad (16)$$

which yields the well known **Cheng-Prusoff equation** for competitive inhibition (Cheng and Prusoff, 1973):

$$K_i = \frac{K_M}{K_M + S} \cdot IC50. \quad (17)$$

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, 1973).

## 2.4 Central relation between time-dependent IC50(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 Eq. (8), since under these two assumptions,  $\eta_I$  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 Eq. (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

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

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

$$P_{IC50}(t) = \frac{V_{\max} \cdot S}{K_M \left(1 + \frac{IC50(t)}{K_I}\right) + S} \left( \frac{1 - e^{-k_{inact} \cdot \eta_{IC50} \cdot t}}{k_{inact} \cdot \eta_{IC50}} \right). \tag{21}$$

with  $\eta_{IC50}$  defined by (9) with  $I=IC50$ . Exploiting Eq. (12) we obtain

$$\frac{1}{2} = \frac{P_{IC50}(t)}{P_0(t)} = \frac{K_M + S}{K_M \left(1 + \frac{IC50}{K_I}\right) + S} \cdot \frac{1}{t} \cdot \left( \frac{1 - e^{-k_{inact} \cdot \eta_{IC50} \cdot t}}{k_{inact} \cdot \eta_{IC50}} \right). \tag{22}$$

This finally yields the central relation between time-dependent  $IC50(t)$  values and the parameters  $K_I$  and  $k_{inact}$ .

$$IC50(t) = K_I \left(1 + \frac{S}{K_M}\right) \cdot \left( \frac{2 - 2e^{-\eta_{IC50} k_{inact} t}}{\eta_{IC50} \cdot k_{inact} \cdot t} - 1 \right) \quad \text{with} \quad \eta_{IC50} = \frac{IC50(t)}{K_I \left(1 + \frac{S}{K_M}\right) + IC50(t)}. \tag{23}$$

The above equation illustrates that there is a specific curve of  $IC50$  values over time for every combination of  $K_I$  and  $k_{inact}$  (see Figure 1). In a typical experimental setup the parameters  $S$ ,  $K_M$ , and  $I$ ,

as well as a series of measured time-dependent IC50 values IC50(1), ..., IC50(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 Eq. (23), e.g., 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 IC50 values. In order to compute the right hand side, the input parameter  $\eta_{IC50}$  is determined based on the measured IC50 values. As a result, the measured IC50 values enter twice in the process of estimating  $K_I$  and  $k_{inact}$ : As an input to the right hand side (via  $\eta_{IC50}$ ), and as experimental data to fit the prediction of the model in Eq. (23). As is well known, the precision of the estimated parameters typically increases with the number of experimental data points (IC50 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 paper.

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

$$K_I = \left( \frac{K_M}{K_M + S} \right) \cdot IC50(t) \cdot C(k_{inact}, t, \eta_{IC50}), \quad (24)$$

where the factor C depends on  $k_{inact}$ , time, and  $\eta_{IC50}$ :

$$C(k_{inact}, t, \eta_{IC50}) = \left( \frac{2 - 2e^{-\eta_{IC50} \cdot k_{inact} \cdot t}}{\eta_{IC50} \cdot k_{inact} \cdot t} - 1 \right)^{-1}. \quad (25)$$

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 Eq. (17). In previous attempts this factor has never been accounted for correctly (e.g., Maurer et al., 2000).

## Results

The derived central relation (23) between  $K_I$ ,  $k_{\text{inact}}$  and the IC50 values was validated *in silico* by generating concentration-time-profiles of the resulting product over 30 minutes using equation (19), see Figure 3 (left). Based on these data, theoretical IC50 values were calculated at each minute based on eq. (12), and used to validate relation (23). In addition, we show the prediction based on the relation (B3) derived in (Maurer et al., 2000). As shown in Figure 3 (right) the herein derived relation is in excellent agreement with the theoretical IC50 values. In contrast, the relationship derived by Maurer et al. differs substantially from the IC50 values, in particular it predicts negative IC50 values.

The performance of the proposed approach was further evaluated based on fluorimetric assay data of seven well-known MBIs for CYP450 isoforms 1A2 and 3A4. All MBI showed the time-dependent decrease in IC50 values (see Figure 4). Clearly, the model predictions nicely describe the decrease of IC50 values due to mechanism based inhibition for all six inhibitors (see Figure 5).

To assess the accuracy of the estimated parameters of inhibition, a comparison to literature values that were obtained in human liver microsomes by use of the Kitz-Wilson or nonlinear regression method is provided. Based on the known parameters  $S$  and  $K_M$ , the inhibition parameters  $K_I$  and  $k_{\text{inact}}$  have been estimated by fitting model (23) to the time dependent IC50 data. In Table 1 the estimated values of  $K_I$  and  $k_{\text{inact}}$  (based on single fluorimetric assay experiments) are compared to values of  $K_I$  and  $k_{\text{inact}}$  estimated by classical data analysis methods (based on a series of pre-incubation experiments). As can be inferred from Table 1, generally there is a good agreement between our estimates and  $K_I$  and  $k_{\text{inact}}$  values based on classical data analysis methods. Calculated  $k_{\text{inact}}$  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, Furofylline and Ethinylestradiol with different sampling protocols were used to assess the impact of experimental variability. Model predictions are in good agreement

with experimental IC<sub>50</sub> values even for small numbers of sampling points (Figure 5). The resulting estimates of  $K_I$  and  $k_{inact}$  for different numbers of sampling point can be found Table 1.

## Discussion

Irreversible inactivation of enzymes by an inhibitor can be both, undesirable when drug metabolising 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 in order to characterize the inhibitory potential. In this article we derive a novel relation to directly estimate these two parameters from time-dependent IC<sub>50</sub> values without the need of a series of measurements with increasing pre-incubation time. The proposed approach is generally applicable to experimental assays that provide a series of time-dependent IC<sub>50</sub> values.

The theoretical derivation is tightly coupled to the experimental conduct of the assay, i.e., the derivation is only based on assumptions, under which the *in vitro* experiment is typically performed. Under these assumptions, Eq. (23) provides a mathematically founded relation between the time-dependence of IC<sub>50</sub> 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 IC<sub>50</sub> value directly to reaction velocities (Maurer et al, 2000). During the performance of the assays, however, product concentrations rather than reaction velocities are measured. Eq. (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 two approaches is apparent from quality of predictions shown in Figure 3. By definition, the velocity of product formation decreases over time 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 realised in our approach



by integrating the velocity over the incubation time, resulting in the term  $\eta_{IC50} \cdot k_{inact} \cdot t$  in the denominator of the central relation (23). A characterization solely based on reaction velocities is questionable.

In order to access the drug-drug interaction potential of new compounds, *in vitro* studies utilizing liver microsomes, hepatocytes or recombinant CYPs are being applied (Venkatakrishnan et al., 2003, Bachmann et al., 2006). 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 magnitude (Venkatakrishnan et al., 2003), e.g. paroxetine and CYP2D6 (Bertelsen et al., 2003), clarithromycin and CYP3A (Mayhew et al., 2000), furanocoumarins and CYP3A (Greenblatt et al., 2003).

As reported by Yan et al., 2002 and Naritomi et al., 2004, irreversible CYP inhibition can easily be distinguished from reversible inhibition by comparing the time-dependent pattern of IC50 values from CYP inhibition experiments. If a fluorimetric assay is used, data for different time points can easily be obtained by reading the fluorescence intensity for an individual plate at several points in time without stopping the enzymatic reactions. Therefore, estimating IC50 values over time using microtiter plate assays is a simple and attractive method to detect irreversible inhibitors (Naritomi et al., 2004). While 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 towards a more quantitative assessment of the DDI potential.

Typically, the experimental determination of  $K_I$  and  $k_{inact}$  values required the performance of additional pre-incubation experiments (Venkatakrishnan et al., 2003), whose data are subsequently analysed by use of the Kitz-Wilson or Non-linear Regression method (Kitz and Wilson, 1962). The presented method, however, allows for the direct estimation of  $K_I$  and  $k_{inact}$  from the set of time-dependent IC50 values.

We have analysed 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 (Bell et al., 2008, Cohen et al., 2003). However, despite these problems, time-dependent fluorimetric assays are used to study compounds with respect to potency of MBI (Atkinson et al., 2005; Ghanbari et al., 2006, Riley et al., 2007).

In order 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, Furofylline and Ethinylestradiol. The good agreement between our model predictions and the experimental IC50 values shows that the derived relation is capable of predicting time-dependent IC50 values as different as shown in Figure 5.

Estimated  $K_I$  values varied roughly 2 fold, while  $k_{inact}$  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., Furofylline, 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 IC50 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 IC50 values and the underlying parameters of inactivation KI and kinact (see, e.g., Berry and Zhao 2008). 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 (Bruno and Njar, 2007).

## **Acknowledgments**

The technical assistance of Jörg Seidler and Ruth Neumeister is gratefully acknowledged.

## **Financial statement**

BK has been supported by the International Max-Planck Research School CBSC, Berlin and Bayer Schering Pharma AG, Berlin.

WH acknowledges financial support by Bayer Schering Pharma AG, Berlin.

## References

- Atkinson A, Kenny JR, and Grime K: Automated assessment of time-dependent inhibition of human cytochrome P450 enzymes using liquid chromatography-tandem mass spectrometry analysis. *Drug Metab Dispos* 2005; 33:1637–1647.
- Bachmann KA: Inhibition constants, inhibitor concentrations and the prediction of inhibitory drug drug interactions: pitfalls, progress and promise. *Curr Drug Metab* 2006; 7: 1-14.
- Bell L, Bickford S, Nguyen PH, Wang J, He T, Zhang B, Friche Y, Zimmerlin A, Urban L, and Bojanic D: Evaluation of Fluorescence- and Mass Spectrometry—Based CYP Inhibition Assays for Use in Drug Discovery. *J Biomol Screen* 2008; 13:343-353.
- Berry LM, Zhao Z: An Examination of IC<sub>50</sub> and IC<sub>50</sub>-Shift Experiments in Assessing Time-Dependent Inhibition of CYP3A4. *Drug Metabolism Letters* 2008; 2:51-59.
- Bertelsen KM, Venkatakrisnan K, von Moltke LL, Obach RS, Greenblatt DJ: Apparent Mechanism-based Inhibition of Human CYP2D6 in Vitro by Paroxetine: Comparison with Fluoxetine and Quinidine. *Drug Metabolism and Disposition* 2003; 31:289-293.
- Bruno RD, Njar VC: Targeting cytochrome P450 enzymes: a new approach in anti-cancer drug development. *Bioorg Med Chem* 2007; 15(15):5047-60.
- Chan WK and Delucchi AB: Resveratrol, a red wine constituent, is a mechanism-based inactivator of cytochrome p450 3a4. *Life Sci* 2000; 67(25):3103-3112.
- Chen Q, Ngui JS, Doss GA, Wang RW, Cai X, DiNinno FP, Blizzard TA, Hammond ML, Stearns RA, Evans DC, Baillie TA, Tang W: Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem Res Toxicol* 2002; 15(7):907-914.
- Cheng HC: The influence of cooperativity on the determination of dissociation constants: examination of the cheng-prusoff equation, the scatchard analysis, the schild analysis and related power equations. *Pharmacol Res* 2004; 50(1):21-40.
- Cheng Y, Prusoff WH: Relationship between the inhibition constant (k<sub>1</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (IC<sub>50</sub>) of an enzymatic reaction. *Biochem Pharmacol* 1973; 22(23):3099-3108.
- Cohen LH, Remley MJ, Raunig D, Vaz AND: In vitro drug interactions of cytochrome P450: an evaluation of fluorogenic to conventional substrates. *Drug Metab Dispos* 2003; 31(8):1005-1015
- Clarke SE, Ayrton AD, Chenery RJ: Characterization of the inhibition of P450 1A2 by furafylline. *Xenobiotica*

- 1994; 24(6):517-526. Crespi CL, Miller VP, Penman BW: Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* 1997; 248(1):188-190.
- Di L, Kerns EH, Li SQ, Carter GT: Comparison of cytochrome P450 inhibition assays for drug discovery using human liver microsomes with LC-MS, rhCYP450 isozymes with fluorescence, and double cocktail with LC-MS. *Int J Pharm* 2007; Apr 20;335(1-2):1-11.
- Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT, Rostami-Hodjegan A: A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Current Drug Metabolism* 2006; 7(3):315-34.
- Greenblatt DJ, von Moltke LL, Harmatz, JS, Chen G, Weemhoff JL, Jen C, Kelley CJ, LeDuc BW, and Zinny MA: Time course of recovery of cytochrome p450 3A function after single doses of grapefruit juice. *Clinical Pharmacology and Therapeutics* 2003; 74:121-129.
- He K, Iyer KR, Hayes RN, Sinz MW, Woolf TF, and Hollenberg PF: Inactivation of cytochrome P450 3A4 by bergamottin, a component of grapefruit juice. *Chem Res Toxicol* 1998; 11(4):252-259.
- He K, Woolf TF, and Hollenberg PF: Mechanism-based inactivation of cytochrome P-450-3A4 by mifepristone (ru486). *J Pharmacol Exp Ther* 1999; 288(2):791-797.
- Kalgutkar AS, Obach RS, Maurer TS: Mechanism-based inactivation of cytochrome p450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. *Curr Drug Metab* 2007; 8(5):407-447.
- Kim DH and Mobashery S: Mechanism-based inhibition of zinc proteases. *Curr Med Chem* 2001; 8(8):959-65.
- Kitz R and Wilson IB: Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J Biol Chem* 1962; 237:3245-3249.
- Kunze KL and Trager WF: Isoform-selective mechanism-based inhibition of human cytochrome P450 1A2 by furafylline. *Chem Res Toxicol* 1993; 6(5):649-656. (2007) vol. 12 (1) pp. 92-9
- Krippendorff BF, Lienau P, Reichel A, and Huisinga W: Optimizing Classification of Drug-Drug Interaction Potential for CYP450 Isoenzyme Inhibition Assays in Early Drug Discovery, *J Biomol Screen* 2007; 12 (1):92-99.
- Lin H, Kent UM, and Hollenberg PF: Mechanism-based inactivation of cytochrome P450 3A4 by 17 alpha-ethynylestradiol: evidence for heme destruction and covalent binding to protein. *J Pharmacol Exp Ther* 2002; 301(1):160-167.
- Maurer TS, Tabrizi-Fard MA and Fung HL: Impact of mechanism-based enzyme inactivation on inhibitor potency: implications for rational drug discovery. *J Pharm Sci* 2000; 89 (11): 1404-1414.
- Mayhew BS, Jones DR, and Hall SD: An In Vitro Model for Predicting In Vivo Inhibition of Cytochrome P450 3A4 by

Metabolic Intermediate Complex Formation. *Drug Metabolism and Disposition* 2000; 28(9):1031-1037.

Myers JK and Widlanski TS: Mechanism-based inactivation of prostatic acid phosphatase. *Science* 1993; 26; 262(5138):1451-3.

Naritomi Y, Teramura Y, Terashita S, and Kagayama A: Utility of microtiter plate assays for human cytochrome p450 inhibition studies in drug discovery: application of simple method for detecting quasi-irreversible and irreversible inhibitors. *Drug Metab Pharmacokinet* 2004; 19(1):55-61.

Obach RS, Walsky RL, and Venkatakrishnan K: Mechanism based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. *Drug Metab Dispos* 2007;35:246–255.

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB, and Tremaine LM: The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J Pharmacol Exp Ther* 2006; 316(1):336-348.

Polasek TM, Elliot DJ, Lewis BC, and Miners JO: Mechanism-based inactivation of human cytochrome P450 2C8 by drugs in vitro. *J Pharmacol Exp Ther* 2004; 311(3):996-1007.

Proctor NJ, Tucker GT, Rostami-Hodjegan A: Predicting drug clearance from recombinantly expressed CYPs: intersystem extrapolation factors. *Xenobiotica* 2004; 34(2):151-78.

Riley RJ, Grime K, and Weaver R: Time-dependent CYP inhibition. *Expert Opin. Drug Metab. Toxicol* 2007; 3(1):51-66.

Scott EE, Halpert JR: Structures of cytochrome P450 3A4. *Trends Biochem Sci* 2005; 30(1):5-7.

Silverman RB: Mechanism-based enzyme inactivators. *Methods Enzymol* 1995, 249:240-283.

Tucker GT, Houston JB, Huang, SM: EUFEPS conference report. Optimising drug development: strategies to assess drug metabolism/transporter interaction potential - towards a consensus. *Eur. J. Pharm. Sci* 2001; 13:417-28.

Turpeinen M, Korhonenb LE, Tolonen A, Uusitalo Jouko, Juvonenb R, Raunio H and Pelkonen O: Cytochrome P450 (CYP) inhibition screening: comparison of three tests. *Eur. J. Pharm. Sci* 2006; 29:130-138.

Venkatakrishnan K, von Moltke LL, Obach RS, and Greenblatt DJ: *Drug Metabolism and Drug Interactions: Application and Clinical Value of In Vitro Models*, *Current Drug Metabolism* 2003; 4: 423-459.

Venkatakrishnan K, Obach RS, and Rostami-Hodjegan A: Mechanism-based inactivation of human cytochrome P450 enzymes: strategies for diagnosis and drug-drug interaction risk assessment, *Xenobiotica* 2007; 37: 1225-1256.

Yamano K, Yamamoto K, Katashima M, Kotaki H, Takedomi S, Matsuo H, Ohtani H, Sawada Y, and Iga T: Prediction of midazolam-CYP3A inhibitors interaction in the human liver from in vivo/in vitro absorption, distribution, and metabolism data. *Drug Metab Dispos* 2001; 29(4 Pt 1):443-452.

- Yan Z, Rafferty B, Caldwell GW, and Masucci JA: Rapidly distinguishing reversible and irreversible CYP450 inhibitors by using fluorimetric kinetic analyses. *Eur J Drug Metab Pharmacokinet* 2002; 27(4):281-287.
- Yang J, Jamei M, Yeo KR, Tucker GT, and Rostami-Hodjegan A: Kinetic values for mechanism-based enzyme inhibition: assessing the bias introduced by the conventional experimental protocol. *Eur J Pharm Sci* 2005, 26(3-4):334-340.
- Yeo KR and Yeo WW: Inhibitory effects of verapamil and diltiazem on simvastatin metabolism in human liver microsomes. *Br J Clin Pharmacol* 2001, 51(5):461-470.
- Zlokarnik G, Grootenhuis PDJ and Watson JB: High throughput P450 inhibition screens in early drug discovery. *Drug Discov Today* 2005; 10(21):1443-1450

## Legends

Table 1: Comparison of  $K_I$  and  $k_{inact}$  values calculated by the herein proposed method and literature data. 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, while 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 in section 2. Literature data are based on human liver microsomes. Units:  $K_I$  in  $\mu\text{M}$  and  $k_{inact}$  in  $\text{min}^{-1}$ .

Figure 1 : Illustration of the influence of different combinations of  $K_I$  and  $k_{inact}$  on the time dependence of the  $\text{IC}_{50}$ . Given a series of  $\text{IC}_{50}$  values over time, this in turn allows to determine the  $K_I$  and  $k_{inact}$  values by a least squares fit using Eq. (23).

Figure 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  $\text{IC}_{50}$  values against incubation time. C and D. Fitting of the model to the  $\text{IC}_{50}$  values (e.g. by using XLfit) to obtain estimates of  $K_I$  and  $k_{inact}$ .

Figure 3: Validation of equation (23) and comparison with the relation derived in (Maurer et al., 2000). Left: Using equation (19), product formulation over 30 minutes was simulated with increasing concentrations of a mechanism-based inhibitor from 0  $\mu\text{M}$  (top) to 9  $\mu\text{M}$  (bottom). The parameters  $V_{max}$ ,  $K_m$ ,  $K_I$ ,  $S$  and  $k_{inact}$  were set to 100  $\mu\text{M}/\text{min}$ , 2.5  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 5  $\mu\text{M}$ , and 0.5  $\text{min}^{-1}$ , respectively (as in Maurer et al.). Right: Comparison of theoretical  $\text{IC}_{50}$  values

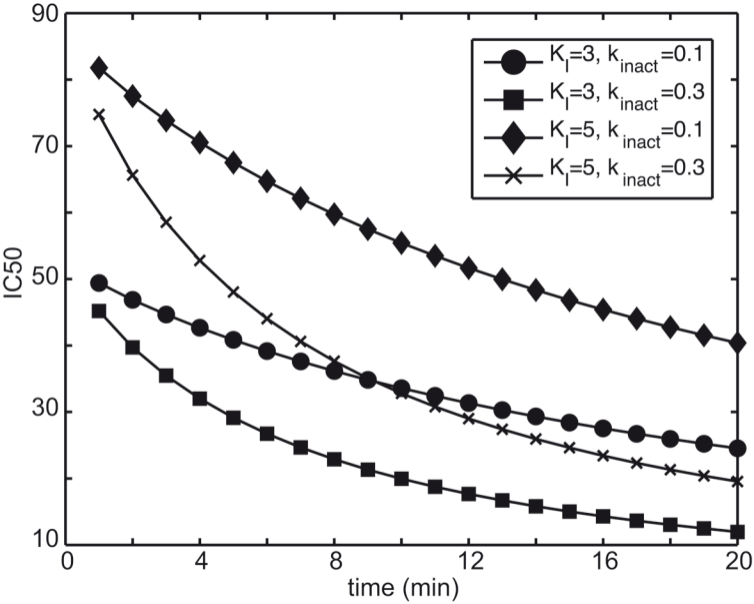


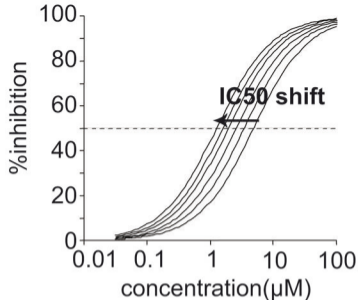
(solid line) with predictions resulting from the herein proposed relation eq. (23) (circles) and the alternative relation (eq. (B3) in Maurer et al. 2000) (squares).

Figure 4: Measured inhibition curves for the seven mechanism-based CYP inhibitors (including replicates) with different inhibitor concentrations and at different points in time. IC<sub>50</sub> 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<sub>50</sub> values have been used subsequently to estimate  $K_I$  and  $k_{inact}$  values for each inhibitor.

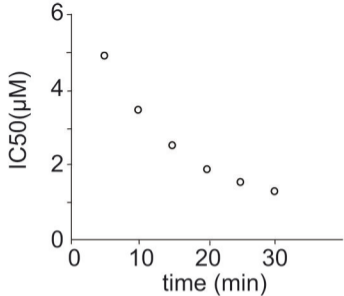
Figure 5: Illustrative time-dependent IC<sub>50</sub> values from CYP P450 inhibition experiments for seven different mechanism-based inhibitors of CYP isoforms 3A4 and 1A2. IC<sub>50</sub> values have been determined at different points in time. For each set of IC<sub>50</sub> values a curve is fitted by using the here developed central relation (23), which represents a specific  $K_I$  and  $k_{inact}$  value with goodness of fit given as normalized root mean square error (RMSE). The obtained values are listed in Table 1. IC<sub>50</sub> values are given in  $\mu\text{M}$ . Each plot is an example of up to four individual experiments.

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)	testosterone, paclitaxel
Furafylline	1A2		X		X	(Clarke et al., 1994; Kunze and Trager, 1993)	warfarin
Erythromycin	3A4		X		X	(Yamano et al., 2001; Chan and Delucchi, 2000)	midazolam, testosterone
Ethinylestradiol	3A4		X		X	(Lin et al., 2002)	testosterone
Bergamottin	3A4		X		X	(He et al., 1998)	testosterone
Raloxifene	3A4		X		X	(Chen et al., 2002)	testosterone





A



B

Solve the following least squares problem by using a software tool (e.g. XLfit)

$$\min |z - f(t,y;p)|$$

with

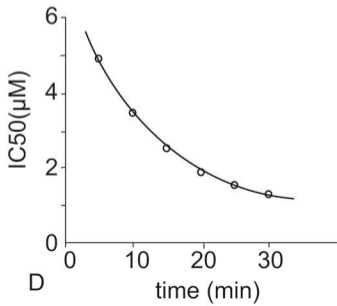
$t = \text{time}$

$y = z = \text{measured IC50}$

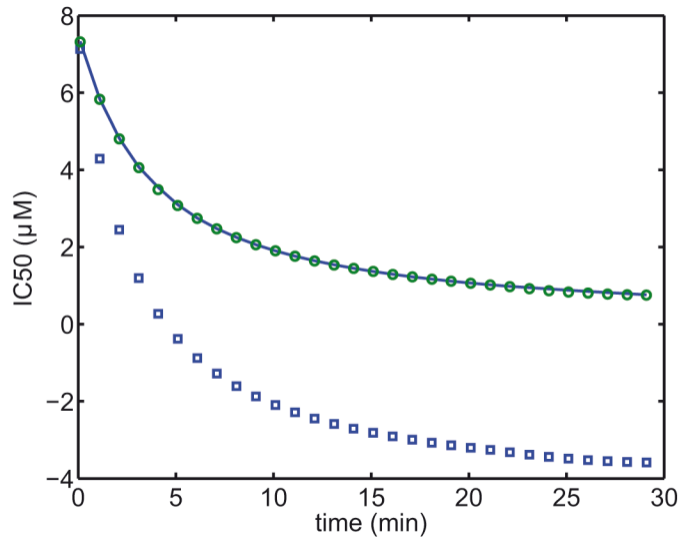
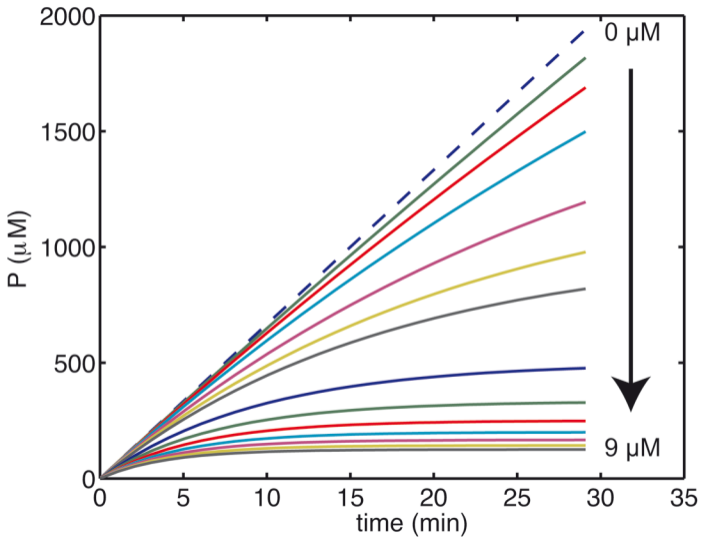
$p = K_I, k_{\text{inact}}$

$f$  - predicted IC50 as def. in eq. (23)

C

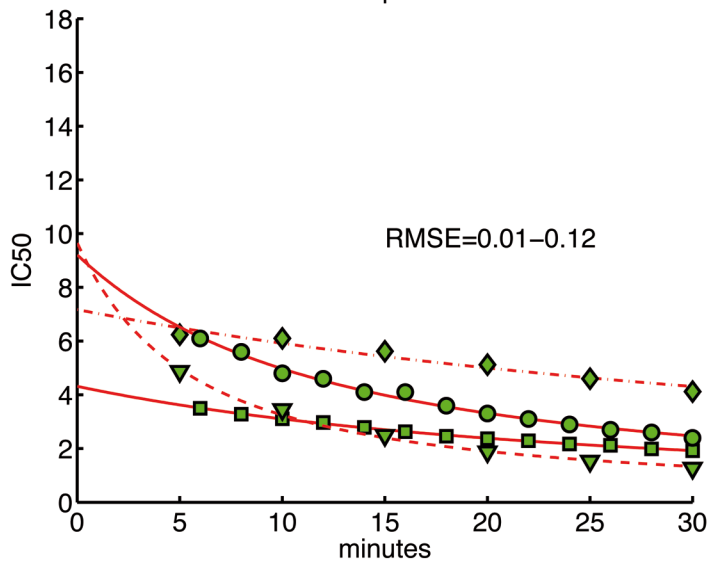


D

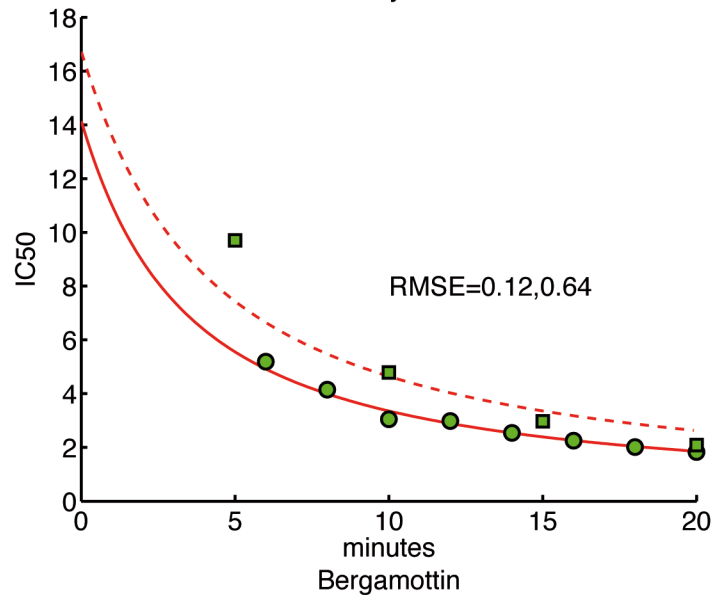




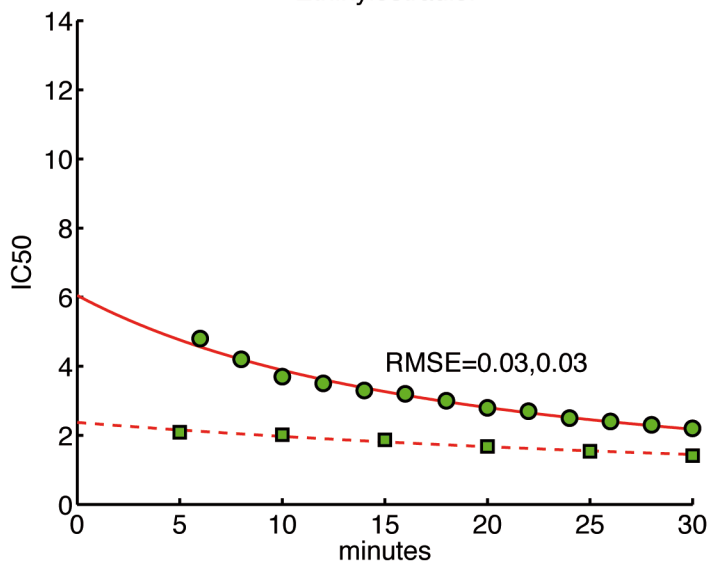
Verapamil



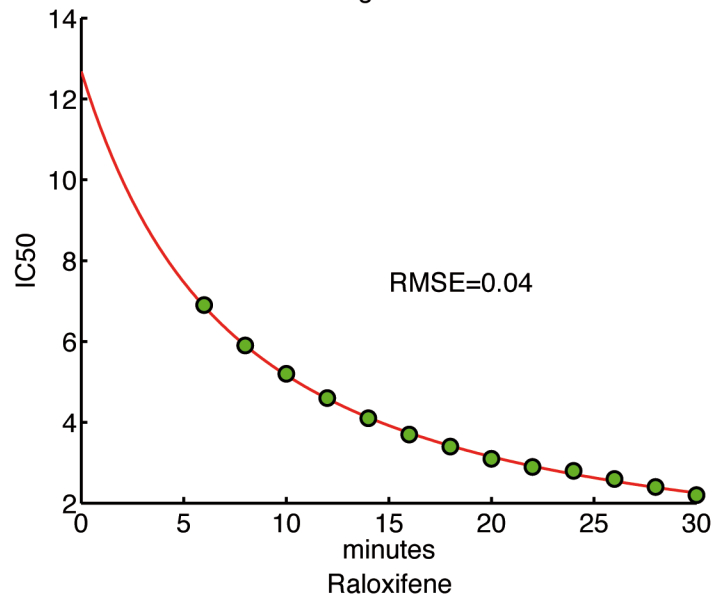
Furafylline



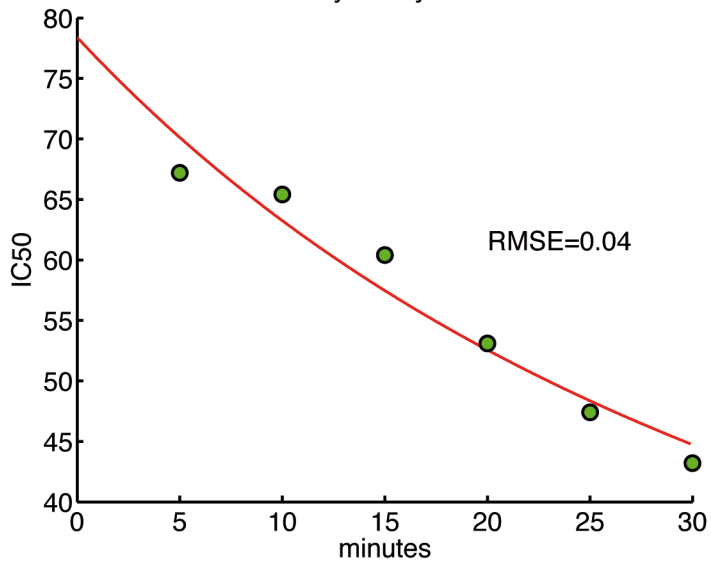
Ethinylestradiol



Bergamottin



Erythromycin



Raloxifene

