# An alternative method for the determination of $K_i$ and $k_{inact}$ parameters for irreversible inhibitor profiling

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## 1 INTRODUCTION

- Over recent years there has been a resurgence in the focussed development of irreversible inhibitors that act via a covalent, time-dependent mechanism. Traditionally the potential for enzyme inhibition has been determined by steady state affinity measurements and quantified in terms of $IC_{50}$. However this parameter in isolation may be misleading when applied to ranking different covalent compounds for structure-activity-relationship studies.

- It has been proposed that in addition to $IC_{50}$, the rate of covalent modification should be taken into consideration (Strelow, 2017). This is defined by the rate constant $k_{inact}/K_i$ where $K_i$, the inhibition constant describes the potency of the first reversible binding event and $k_{inact}$ the maximum rate in inactivation.

- A commonly used technique is to derive $k_{inact}/K_i$ by plotting the observed rate of inactivation ($k_{obs}$) as a function of inhibitor concentration. Other methods employ an approach based on the effect of time on $IC_{50}$ which typically involves many pre-incubation steps. In 2009 Krippendorff et al described a novel alternative to this whereby the pre-incubation step is omitted and data is fitted to a 3D model in IDBS ActivityBase™.

- At Charles River we have established a capability which utilises the Krippendorff time-dependent $IC_{50}$-based method in $k_{inact}/K_i$ studies. Here, we present a comparative study where we applied standard time-course/$k_{obs}$ fitting with the time-dependent $IC_{50}$ method using data generated from a recombinant CYP3A4 system.

## 2 EXPERIMENTAL

- This study was based on the work detailed by Krippendorff et al.,2009. We used a commercially available recombinant CYP3A4 fluorogenic assay (Vivid® CYP3A4 Blue, Life Technologies™, P2858) to kinetically profile a panel of well characterised time-dependent CYP 3A4 inhibitors. The assay measures CYP3A4-mediated oxidation of a BOMCC substrate which liberates fluorescent metabolites that are excited in the visible spectrum. The amount of fluorescent signal is directly proportional to the amount of CYP3A4 activity.

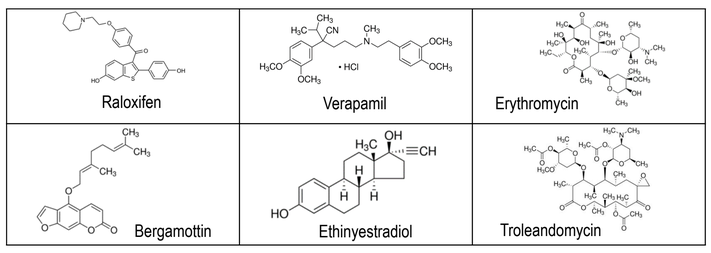


**Table 1:** Time-dependent CYP3A4 inhibitors

1. Compounds were assayed in duplicate as a 20 point 2-fold serial dilution with a top concentration of 100 µM.
2. The reaction was initiated by the addition of CYP3A4/reductase regeneration system to a mixture of NADP, BOMCC substrate and compounds in kit assay buffer. Data was collected on the Tecan Safire II at 180s intervals for 30 minutes (Excitation=415nm, Emission =460nm).

## 3 DATA ANALYSIS

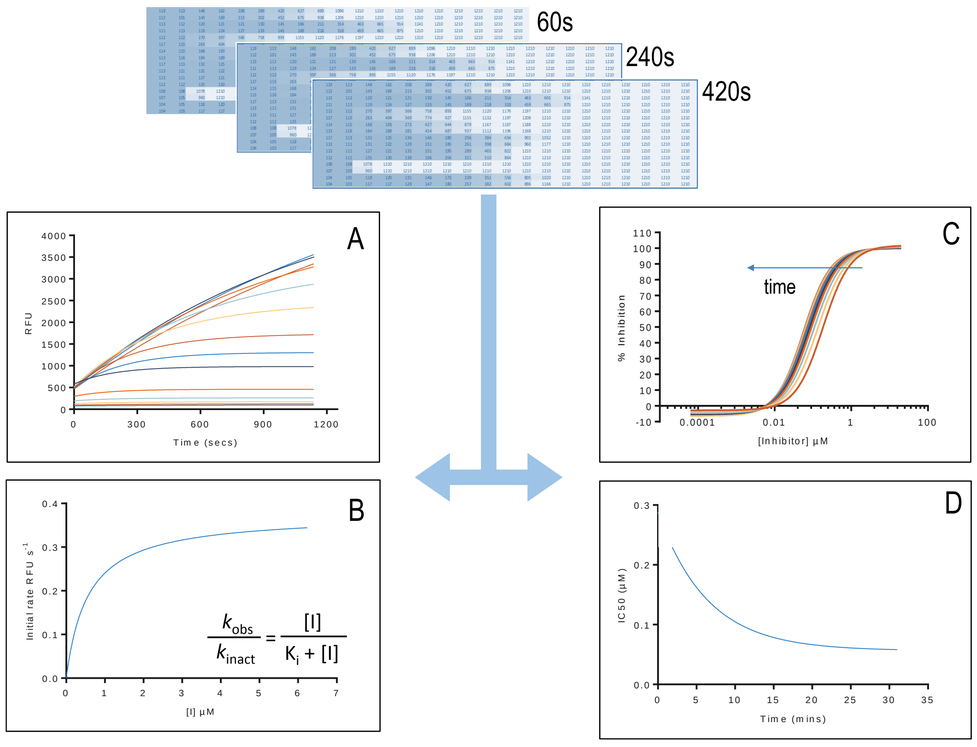


**Figure 1: Schematic of the approach to data analysis**. A) Time-course data. B) Secondary plot of $k_{obs}$ vs. [I]. C) $IC_{50}$ curves at various timepoints. D) Plot of $IC_{50}$ vs. time. Application of the XLFit template significantly reduces the data turn around time

**1.** Kinetic datasets were analysed by fitting the time-course data to a single exponential to derive $k_{obs}$ for each inhibitor concentration. A secondary plot of $k_{obs}$ vs. [I] was used to determine $k_{inact}$ and $K_i$ (equation inset). All analyses were carried out in Graphpad Prism.

**2.** The same raw datasets were normalised and used to produce inhibition curves at each timepoint in order to generate $IC_{50}$ values using a 4 parameter fit model with a Huber robust fitting algorithm. The values were plotted against incubation time and the Krippendorff model fitted to obtain $K_i$ and $k_{inact}$ estimates, using XLFit and IDBS ActivityBase™.

## 4 RESULTS

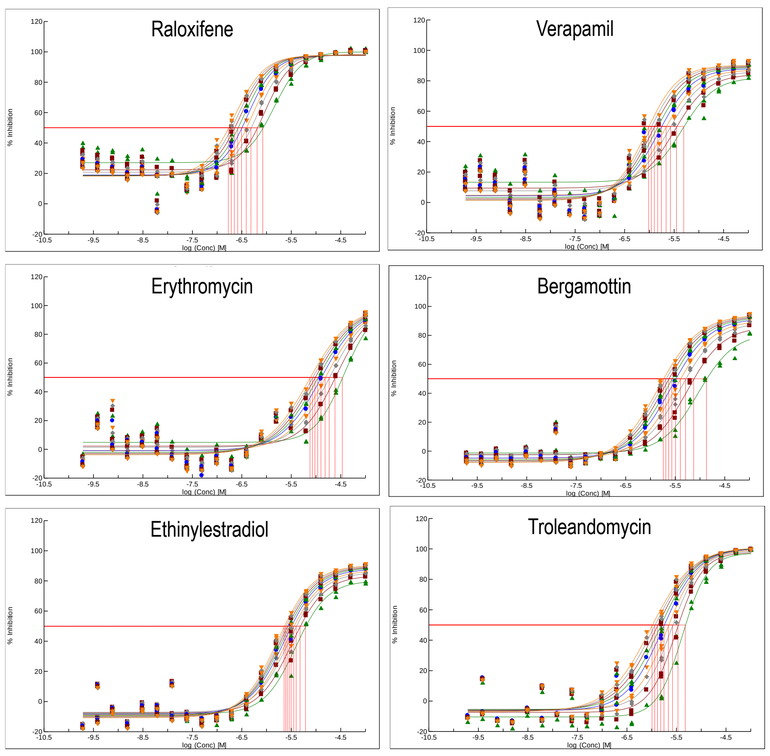


**Figure 2: XLFit $IC_{50}$ curve profiles for the six time-dependent CYP3A4 inhibitors.** Data generated between 3.5 and 27.5 minutes. Dotted red lines indicate the point of inflection at each timepoint. All curves show a leftward shift with time.

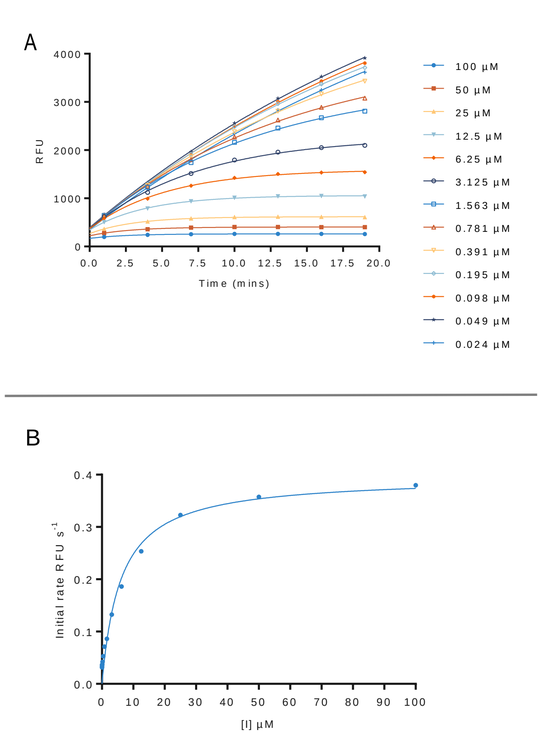


**Figure 3: Graphpad Prism Erithromycin inhibition fitting using $k_{obs}$.** A) Time-course data. B) Secondary plot of $k_{obs}$ vs [I]

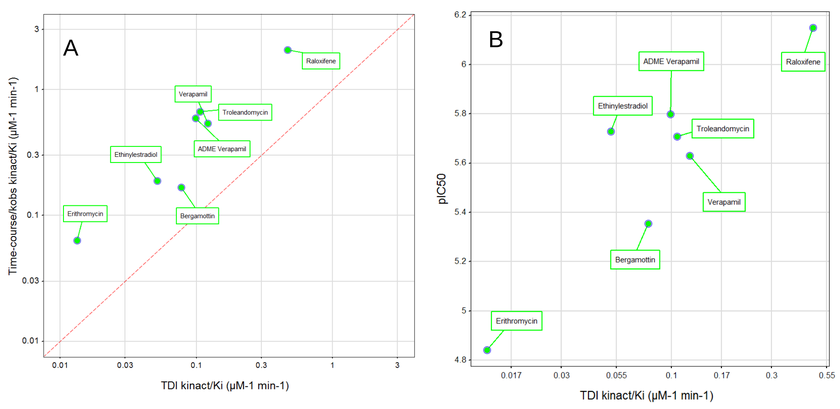


**Figure 4: Correlation plots.** A) $k_{inact}/K_i$ ratios determined using $k_{obs}$ (*y*-axis) or time-dependent $IC_{50}$ fitting (*x*-axis). B) The relationship between pIC50 and $k_{inact}/K_i$ derived using time-dependent $IC_{50}$ values

| CYP3A4 Inhibitor | $K_i$ (µM)* | $k_{inact}$ min-1* | $k_{inact}/K_i$ (min-1 µM-1)* | $K_i$ (µM)† | $k_{inact}$ min-1† | $k_{inact}/K_i$ (min-1 µM-1)† | $IC_{50}$ (µM) @3.5 minutes | $IC_{50}$ (µM) @27.5 minutes | Literature $K_i$ (µM) |
|---|---|---|---|---|---|---|---|---|---|
| Raloxifene | 2.1 | 1.004 | 0.470 | 0.235 | 0.487 | 2.073 | 0.708 | 0.115 | 9.9 |
| Verapamil | 5.0 | 0.610 | 0.122 | 0.392 | 0.211 | 0.538 | 2.343 | 0.495 | 4.2 |
| Erithromycin | 29.9 | 0.398 | 0.013 | 6.435 | 0.408 | 0.063 | 14.402 | 3.881 | 81.8 |
| Bergamottin | 15.8 | 1.225 | 0.078 | 1.708 | 0.284 | 0.166 | 4.411 | 0.719 | 7.7 |
| Ethinylesradiol | 2.2 | 0.116 | 0.052 | 0.600 | 0.113 | 0.188 | 1.867 | 0.799 | 18.0 |
| Troleandomycin | 3.6 | 0.388 | 0.106 | 0.561 | 0.375 | 0.669 | 1.955 | 0.435 | 1.8 |

**Table 2**: Comparison of time-dependent inhibitor kinetic parameters calculated by time-dependent $IC_{50}$ (*) or time-course/$k_{obs}$ (†) methods and compared to literature. Literature $K_i$ values are based on human liver microsomes.

## 5 CONCLUSIONS

- The time-dependent $IC_{50}$ fitting method in ActivityBase XLFit facilitated more efficient data analysis compared to the time-course/$k_{obs}$ approach in Graphpad Prism and allowed $K_i$ and $k_{inact}$ parameters for six CYP3A4 inhibitors to be calculated within 30 minutes.

- In most cases we found good agreement between the two fitting methods in terms of the $k_{inact}$ parameter however there was a difference of up to one order of magnitude with respect to the $K_i$. Despite this, the test compounds showed the same rank order with respect to the $k_{inact}/K_i$ ratio.

- When compared to literature $K_i$ values we found those derived using time-dependent $IC_{50}$ readout to be closest with all compounds apart from Ethinyestradiol being within 3-fold of the human liver microsomes results

- The Krippendorff method is currently being utilised on a number of projects at Charles River and is proving to be an effective method for irreversible inhibitor profiling

**References:** B.-F. Krippendorff, R. Neuhaus, P. Lienau, A. Reichel, W. Huisinga, Mechanism-based Inhibition: Deriving $K_i$ and $k_{inact}$ directly from Time-Dependent IC50 Values, J Biomol Screen. 14 (2009), pp. 913-923
M Strewlow, A Perspective on the Kinetics of Covalent and Irreversible Inhibition, SLAS Discov., 1 (2017), 3-20