Methods to analyse Mpro time-course data for irreversible inhibitors

Wal Ward
Walter Ward Consultancy & Training
http://www.linkedin.com/in/walterward; wal.ward@hotmail.co.uk

Summary

Nir London, Haim Barr and colleagues generated experimental data for inhibition of the main protease (Mpro) of SARS-Cov2 by chloroacetamide CVD-0000654-001 and acrylamide CVD-0001870-001. I describe how the data have implications for the characterisation of irreversible inhibitors. I suggest requirements for experimental design and steps for data analysis. Compound 654 gives k_{inact} = 0.0024 s^{-1} and K_i = 33 μM (k_{inact}/K_i as 73 s^{-1} M^{-1}). The half-life for Mpro inactivation is around 4.8 min at saturating inhibitor. For compound 1870, it was not possible to estimate separate values for k_{inact} and K_i. The ratio k_{inact}/K_i is 0.88 s^{-1} M^{-1}, which is 83-fold lower than for compound 654. Suggestions are made for further experiments.

Introduction

Assays were started by addition of 5 nM Mpro to 375 nM fluorescent substrate (approximate K_m 40 μM) and data were collected at 2 min intervals for 238 min in a variant of the method posted online. Validity of conventional kinetic theory is dependent upon the assay being at steady-state with respect to concentrations of free inhibitor and substrate (see Morrison & Walsh, 1988; Copeland, 2005). Although the enzyme concentration is only 75-times that of the substrate, the assay seems to contain sufficient substrate, possibly because the enzyme is dimeric with a K_d around 2.5 μM (Zhang et al, 2020) and so largely dissociated into inactive monomer, giving around only 0.039 nM active dimer.

The following eq was fitted to the curved time courses for product accumulation

$$P = c + [v_2 t + (v_1 - v_2)(1 - e^{-k_{obs} t}) / k_{obs}] \quad (1)$$

where v_1 is the initial velocity, v_2 is the non-enzymic velocity, k_{obs} is the observed rate constant for enzyme inactivation and c is the initial offset. The value of k_{obs} depends on inhibitor concentration, according to

$$k_{obs} = k_{inact}\left(\frac{[I]}{K_i + [I]}\right) \quad (2)$$

where k_{inact} is the inactivation rate constant at saturating inhibitor and K_i is the inhibition constant in the scheme

$$E + I \underset{K_i}{\rightleftharpoons} E.I \xrightarrow{k_{inact}} E\text{-}I \quad (3)$$

In general, the observed inhibition constant is higher than the true inhibition value. In these studies, however, assuming inhibition is competitive the observed K_i is close to the true value, because the substrate concentration is well below K_m. In some cases, the initial noncovalent E.I complex cannot be detected, giving the kinetic scheme

$$E \xrightarrow{k_{inact}[I]/K_i} E\text{-}I \qquad (4)$$

where [I] << K_i. Accordingly, eq (2) becomes

$$k_{obs} = \frac{k_{inact}}{K_i}[I] \qquad (5)$$

Observations on experimental data and implications for data analysis

Data were analysed using *Grafit* 3.01 (http://www.erithacus.com/).

At the first time-point, there is a significant signal, which varies between experiments. This "offset" should be estimated for each time course in order to allow characterisation of subsequent kinetics.

No enzyme ("positive") controls show a significant rate (Fig 1). This could be due to non-enzymic hydrolysis or machine drift. These assays can be used to estimate the rate after all of the enzyme has reacted with a covalent inhibitor (v_2). The value of v_2 usually is allowed to vary during curve fitting, but in some cases, it was necessary to constrain v_2 to an average non-enzymic rate (2.52 units/min).

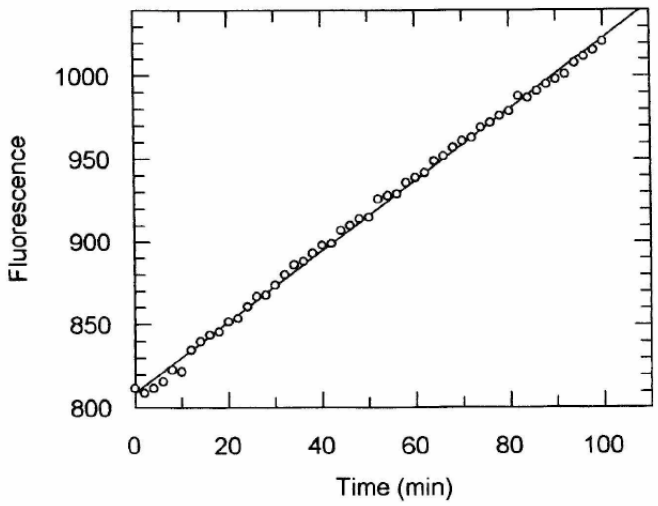

Fig. 1. Time-course in the absence of enzyme. The apparent velocity of 2.14 units/min is estimated by fitting a straight line.

No inhibitor ("negative") controls show a gradual decrease in rate during the 238 min time-course (Fig 2). Rate approximates to linear for the first 60-100 minutes and only this period should be used for data analysis. The subsequent decrease could be due to substrate depletion, product inhibition, enzyme instability or machine drift. Any of these processes invalidates the methods for data analysis, so that curve-fitting should use only the part of the time-course which approximates to

linear. The assay cannot measure precisely observed rate constants for covalent inhibition (k_{obs}) that are slower than the rate constant for loss of activity in the absence of inhibitor. The k_{obs} in the absence of inhibitor was estimated in order to ascertain the minimum value measurable in the presence of inhibitor.

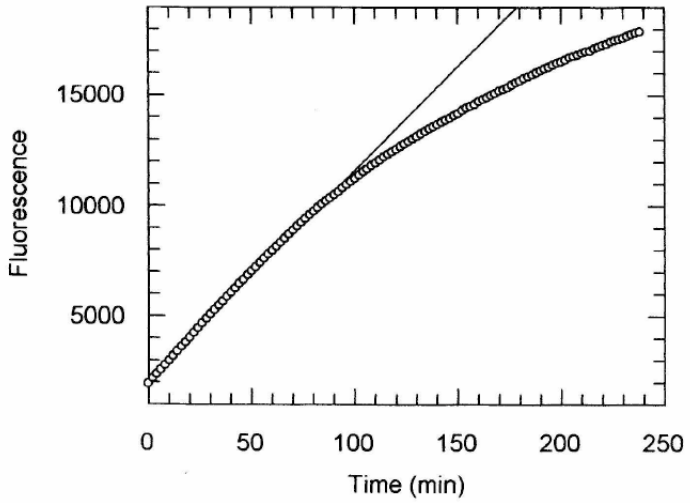

Fig 2. Time-course in the absence of inhibitor. Fitting a straight line to the first 100 min gives a slope of 94 U/min. Curve-fitting using eq (1) with v_2 fixed to 2.52 U/min gives v_1 = 113 U/min, k_{obs} = 0.0039 min^{-1}.

Important requirements for experimental design

1. Assays should be started by addition of enzyme and then a time-course should be collected.
2. Data should be processed only from the period that is close to linear in the absence of inhibitor.
3. Accumulation of product should approximate to linear with time in the absence of inhibitor.
4. Velocity without inhibitor should be linear with [enzyme].
5. Free inhibitor concentration should remain approximately constant during the time-course. It should not decrease due to instability, use as a substrate, nor slow tight binding to the target enzyme. (Analysis of slow tight binding is more complex than the procedures described here and methods are given by Schwartz et al, 2014). "Tight" binding is when free inhibitor is significantly depleted by binding to the target. This does not occur in the current data.

Steps in data analysis

It is important to check there is an acceptable the quality of fit. This can be helped by viewing the residual differences between the experimental data and the calculated best-fit line. The estimated parameter values and standard errors should be reasonable. If not, it may be necessary to constrain variable(s) or fit an alternative equation.

1. Inspect negative (no inhibitor) controls to determine how much of the time-course approximates to linearity (likely to be the first 60-100 minutes, see Fig 2). Fit straight lines to these parts of the data sets to estimate an average the uninhibited rate, v_0

(here around 104 U/min). **All subsequent steps should use only data from the same part of the time-course.**

2. Fit a straight line to the positive controls (no enzyme) to estimate the average rate for non-enzymic change of signal (here approximately 2.52 U/min, see Fig 1). This should be the apparent rate of reaction after full inhibition of the enzyme (v_2).

3. Fit eq (1) to each of the negative (no inhibitor) controls (see Fig 2) to estimate the rate of loss of enzyme activity in the absence of inhibitor (k_{obs} average estimated as 0.0039 min^{-1}). In order to obtain a fit, it may be necessary to constrain the value of v_2 to the average from the no enzyme controls.

4. For data in the presence of inhibitor, fit eq (1) (see Figs 3 & 6). It may be necessary to constrain the value of v_2 as in step 3. Inhibitor k_{obs} values close to those in the absence of inhibitors need to be interpreted with caution, because they are affected by multiple processes.

5. Fit eq (2) to the inhibitor concentration dependence of k_{obs} in order to estimate k_{inact} and K_i (Fig 4). If the range of inhibitor concentrations is below K_i, then the plot approximates to a straight line, with a gradient of k_{inact}/K_i (Fig 7).

This analysis assumes that inhibition does not follow co-operative kinetics. However, most of the posted IC50 data on reversible inhibitors shows positive co-operativity, Hill coefficient (slope) 1.2 to 1.4. It is likely that similar kinetics are followed by irreversible inhibitors. This has not been included in the rate equations used for irreversible inhibition, because it increases uncertainty in parameter values and is unlikely to have a large effect on the rank order of inhibitors (see Fig 4).

Application to data received 04 Jun 2020

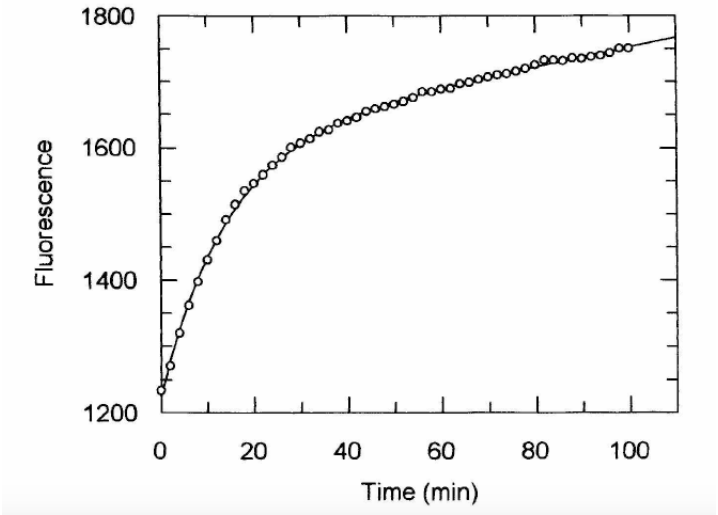

Fig 3. Inactivation by CVD-0000654-01. [Inhibitor] was 32.3 µM, fitting eq (1) estimated v_1 = 29 U/min, v_2 = 1.47 U/min, k_{obs} = 0.072 min^{-1} and offset = 1223. The value of v_1 is less than the uninhibited rate (consistent with detection of an initial noncovalent complex, E.I in eq 3) and v_2 is close to the average non-enzymic rate (consistent with irreversible inactivation of the enzyme).

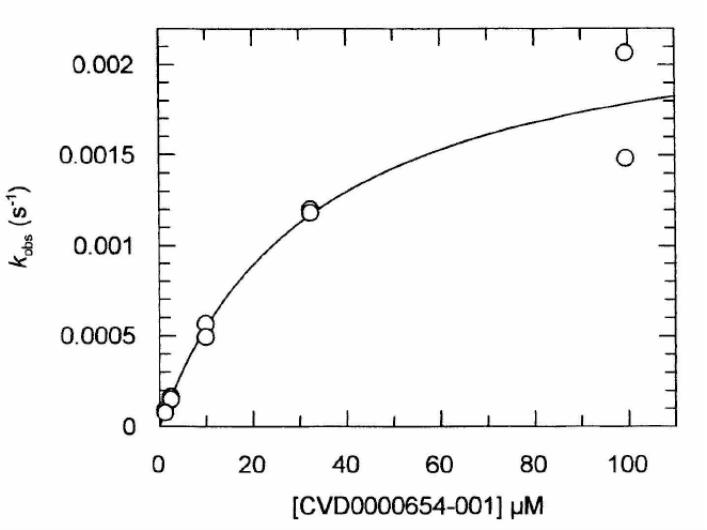

Fig 4. Estimation of k_{inact} and K_i for CVD-0000654-001. Eq (2) was fitted to k_{obs} values estimated using eq (1). The best-fit values are k_{inact} = 0.0024 s^{-1} (95% CIs 0.0018 to 0.0031 s^{-1}) and K_i = 33 µM (95% CIs 17-63), giving k_{inact}/K_i as 73 s^{-1} M^{-1}. The k_{inact} value allows calculation of the half-life for inactivation as 4.8 min at saturating inhibitor. Fitting an equation for co-operative kinetics to these data does not improve the quality of fit, giving a Hill coefficient h = 1.1 (close to h = 1.0 for non co-operative binding) and large standard errors in the estimates for h and K_i.

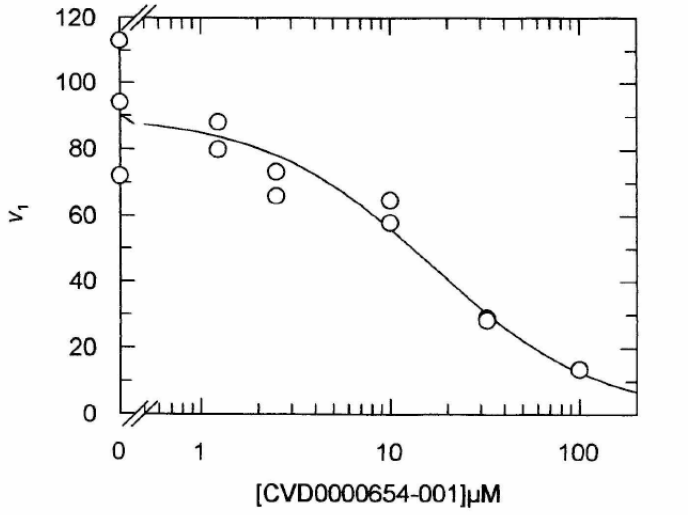

Fig 5. Estimation of K_i from v_1 values. Eq (1) was fitted to estimate initial velocity in the presence of various concentrations of compound 654. The best-fit value of K_i is 17 µM, which is reasonably close to the K_i = 33 µM estimated from the inhibitor-dependence of k_{obs} (Fig 4). Similarly, dose-response analysis of fluorescence measured after 10 min gives IC_{50} = 36 µM, slope 0.46. A slope below 1 may indicate the possibility of slow binding or irreversible inhibition.

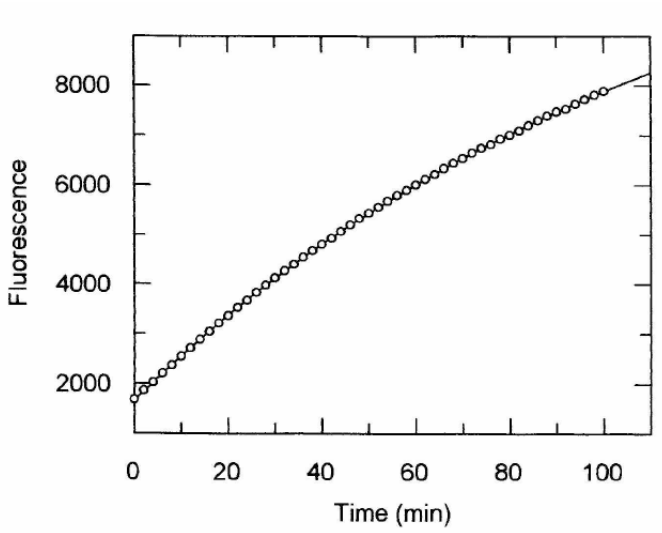

Fig 6. Inactivation by CVD-0001870-01. [Inhibitor] was at 99 μM, fitting eq (1) estimated v_1 = 93 U/min, v_2 = 4.8 U/min, k_{obs} = 0.0094 min^{-1} and offset = 1667. The value of v_1 is similar to the uninhibited rate (consistent with failure to detect an initial noncovalent complex, eq 4) and v_2 is close to the average non-enzymic rate (consistent with irreversible inactivation of the enzyme).

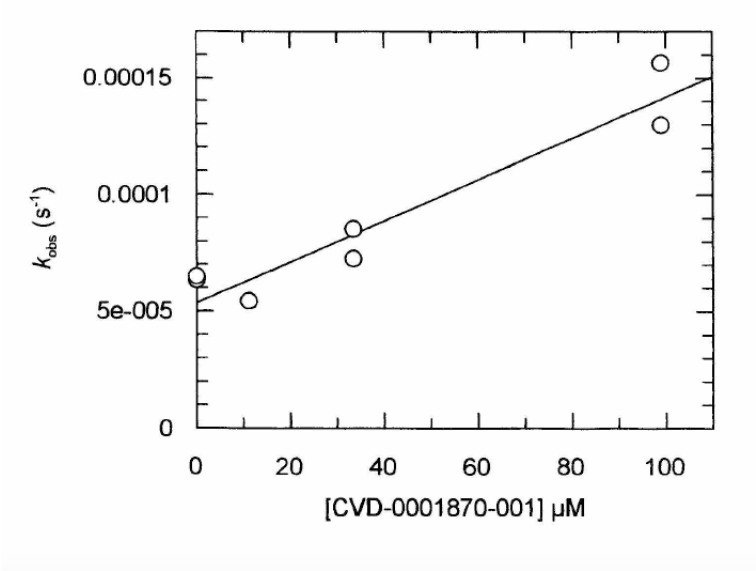

Fig 7. Estimation of k_{inact} and K_i for CVD-0001870-001. Values of k_{obs} at zero inhibitor were estimated from no inhibitor controls. It was not possible to fit eq (2) to k_{obs} values because [inhibitor] is too low relative to K_i (a noncovalent E.I complex was not detected, eq 4). Consistent with this interpretation, it was not possible to estimate K_i from calculated v_1 values. A straight line was fitted for k_{obs} vs [I] (eq 5 plus an offset), giving a gradient k_{inact}/K_i as 0.88 s^{-1} M^{-1} (95% CIs 0.66 to 1.2 s^{-1} M^{-1}) and an offset k_{obs} at zero inhibitor as 5.4 x 10^{-5} s^{-1} (95% CIs 4.2 to 6.9 x 10^{-5} s^{-1}) (0.0032 min^{-1}), which was close to that measured for inactivation of the enzyme in the absence of inhibitor. The k_{inact}/K_i ratio indicates this compound is 83-fold lower than that for compound 654 (Fig 4).

Suggested future work

1. What is the evidence that assays follow Mpro rather than a contaminating enzyme? Is this proved by agreement between crystal structures and enzyme

activity data, or is it necessary to engineer a Cys145Ala active site mutant to show catalytic activity does not arise from a contaminant?

2. Measure if inhibitors react with 1 mM TCEP in order to assess stability during the assay. Rather than develop an assay for each compound, it is possible to monitor formation of oxidized TCEP.

3. It is important to measure the reactivity of covalent inhibitors in 5 mM reduced GSH. This gives insight into stability inside cells and non-specific reactivity towards Cys residues in proteins.

4. Directly determine whether inhibition is irreversible by preincubating enzyme and inhibitor, diluting and then measuring Mpro activity.

5. Mpro potentially could catalyse the conversion of a test compound into a less potent inhibitor (with a rate constant k_p). Effectively the inhibitor would be a substrate, which is inactivated by the enzyme. This can give time-courses which resemble slow binding, reversible inhibition. The partition ratio is defined as k_p/k_{inact} and has a value of 0 if the inhibitor is not used as a substrate. Its value can be estimated by plotting % remaining enzyme activity (steady state, after all inhibitor has been consumed) against the ratio [inhibitor]/[enzyme], which has a negative slope and intercepts the y-axis at 100% and the x-axis at the number of moles of inhibitor required to inactivate 1 mole of enzyme ($=k_p/k_{inact}$) (Copeland, 2005).

Walter Ward 11 June 2020

Walter Ward accepts no liability. Users are responsible for direct & indirect consequences of using this material

References

Copeland RA (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery*, Wiley Interscience, Hoboken New Jersey.

Morrison JF & Walsh CT (1988) The behavior & significance of slow-binding enzyme inhibitors, *Adv Enzymol 61*, 201-301.

Schwartz PA et al (2014) Covalent EGFR inhibitor analysis reveals importance of reversible interactions to potency and mechanisms of drug resistance, *PNAS 111*, 173-178.

Zhang L et al (2020) Crystal Structure of SARS-CoV-2 Main Protease Provides a Basis for Design of Improved α-Ketoamide Inhibitors, *Science 368*, 409-412.