A study for the determination of the mechanism of inhibition (MOI) of ATPase inhibitors

David Whalley & Sophie Harding
Charles River Discovery, Chesterford Research Park, Cambridge, UK

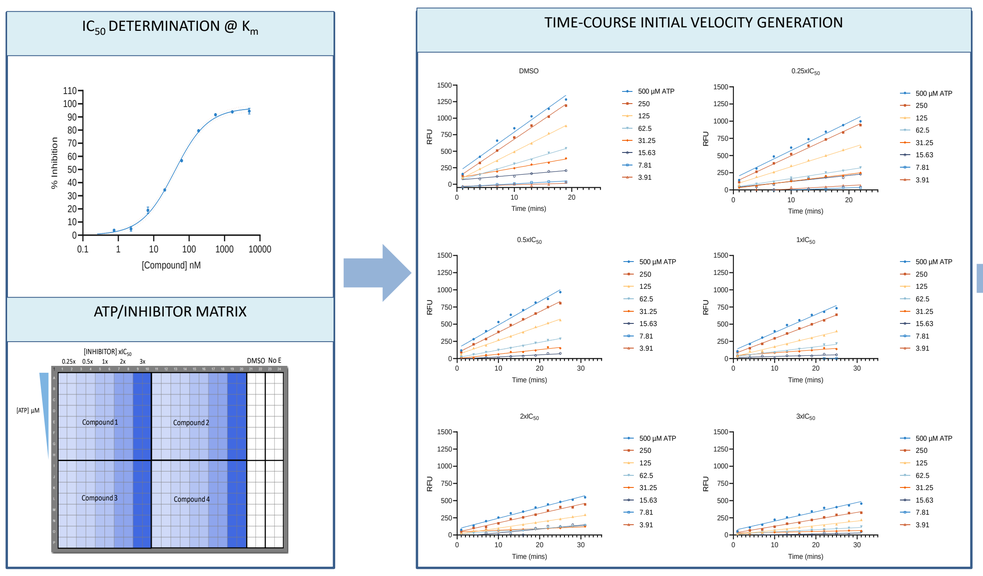
charles river

1 INTRODUCTION

- One third of current drug discovery pipelines are focussed on enzyme drug targets and half of currently marketed drugs are enzyme inhibitors.
- During the initial drug development process, after a high-throughput screening (HTS) campaign of small molecules, hits of interest are further characterised by performing concentration response curve (CRC) studies whereby the potential for enzyme inhibition is determined by steady state affinity measurements and quantified in terms of IC_{50}. This IC_{50} information together with structural analysis defines the structural-activity relationship (SAR) of classes of hit chemical matter.
- Whilst important, the IC_{50} value in isolation is insufficient to describe the compound's mechanism of action (MOA), such as inhibitory mechanism, reversibility, or target residence time. Therefore, MOA studies that historically tended to be positioned at the later phases such as lead optimisation, over recent years have been brought into the screening cascade much earlier.
- Determining the mechanism of inhibition allows the interplay between enzyme, substrate and inhibitor to be understood. Test compounds may possess competitive, non-competitive or uncompetitive mechanisms and defining this could help provide a way of extrapolating the activity seen in biochemical assays to effects seen in whole cells.
- To elucidate the mechanism of inhibition of inorganic phosphate-generating enzyme inhibitors we have established a fluorogenic, real time assay to perform substrate/inhibitor matrix experiments Here, we present a study where we mechanistically characterised a discrete set of ATPase inhibitors.

2 EXPERIMENTAL

For this study we used a commercially available fluorogenic assay (Phosphate sensor, Life Technologies,™, PV4407) to kinetically profile a set of ATPase inhibitors, composed of two specific series. The assay measures ATPase-mediated hydrolysis of ATP by detecting one of the products of the reaction, inorganic phosphate (P_i). Binding of P_i changes the immediate environment of a coumarin (MDCC) fluorophore and gives rise to an increase in fluorescence. The amount of fluorescent signal is directly proportional to the amount of ATPase activity.



PROCEDURE

1. IC50 determinations were carried out by assaying compounds in duplicate as a 12 point 2-fold serial dilutions with a top concentration of 10 µM or 100 µM.

2. For mechanism of inhibition studies compounds were assayed in duplicate at 0.25x, 0.5x 1x, 2x and 3x their respective IC_{50}s, in the presence of 7 ATP concentrations with a top concentration of 500 µM ($10xK_m$)

3. Reactions were initiated by the addition of the ATPase to a mixture of ATP substrate and compounds in assay buffer. Data was collected on the Tecan Safire II at 180s intervals for 30 minutes (Excitation=430nm, Emission=450nm) for both IC50 and MOI experiments.

DATA ANALYSIS

For IC_{50} determinations, raw data taken from a single timepoint during the initial rate of the reaction was normalized and used to produce inhibition curves in order to generate IC_{50} values using a 4 parameter fit model in Graphpad Prism software.

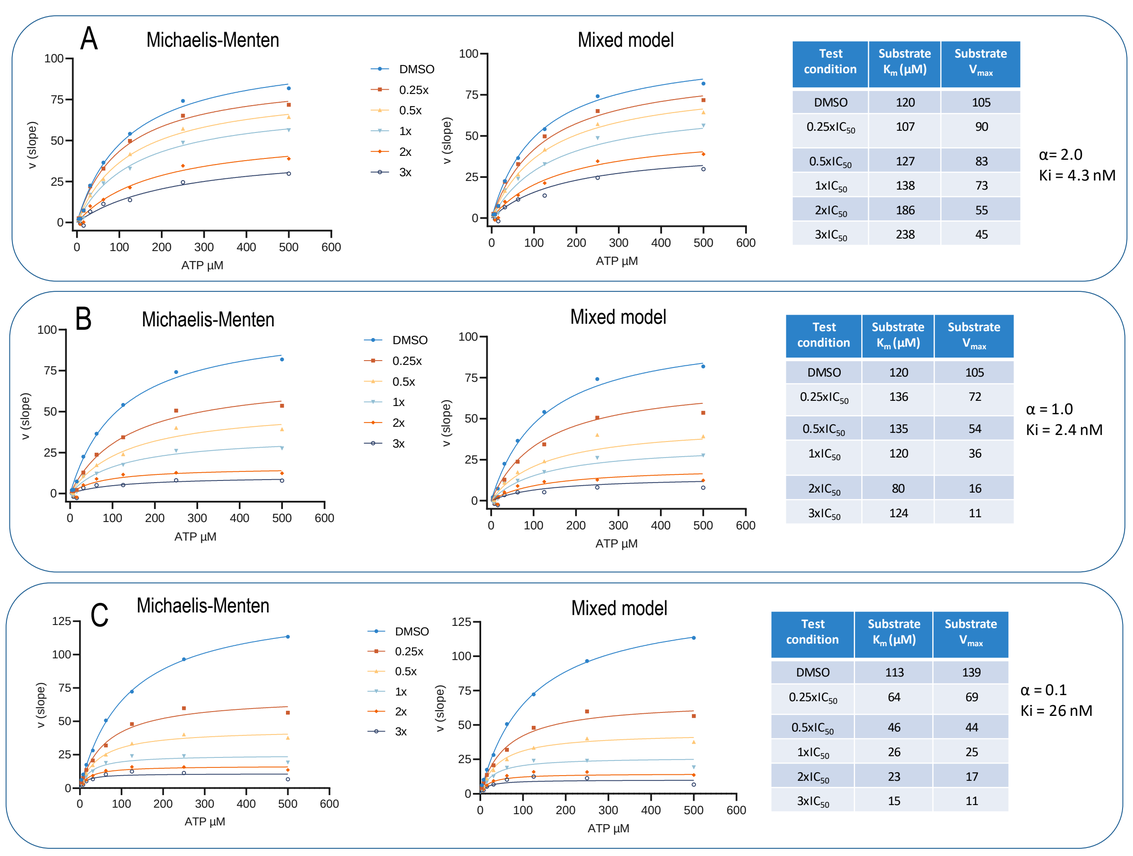
For mechanism of inhibition determinations, kinetic datasets were analysed by calculating the initial rate (v_0) from the time-course data, for each inhibitor concentration at each ATP concentration. A secondary plot of v_0 vs. [ATP] at each inhibitor concentration was used to determine K_m and V_{max} parameters by fitting data to the Michaelis-Menten model or alpha and Ki parameters by fitting data to the general mixed inhibition model. Additional model comparisons were performed by fitting the data either mixed, un-competitive and non-competitive inhibition models. All analyses were performed in Graphpad Prism software.

3 RESULTS

Interpretation of inhibition modalities was done in accordance with that described by Robert Copeland in "Evaluation of Enzyme Inhibitors in Drug Discovery". Inhibition model comparisons showed that compounds possessed either non-competitive or more mixed mechanisms. For data reporting in most cases it was decided to classify compounds as non-competitive and quote the α parameter.

Parameter	Inhibition Modality				
	Competitive	Noncompetitive (α > 1)	Noncompetitive (α = 1)	Noncompetitive (α < 1)	Uncompetitive
K_m	Increases linearly with increasing [I]	Increases curvilinearly with increasing [I]	No effect	Decreases curvilinearly with increasing [I]	Decreases curvilinearly with increasing [I]
V_{max}	No effect	Decreases curvilinearly with increasing [I]	Decreases curvilinearly with increasing [I]	Decreases curvilinearly with increasing [I]	Decreases curvilinearly with increasing [I]

Table 1: Effects of inhibitors of different modalities on values of steady state kinetic parameters



Test condition	Substrate K_m (µM)	Substrate V_{max}
DMSO	120	105
0.25xIC50	107	90
0.5xIC50	127	83
1xIC50	138	73
2xIC50	186	55
3xIC50	238	45

α= 2.0
Ki = 4.3 nM

Test condition	Substrate K_m (µM)	Substrate V_{max}
DMSO	120	105
0.25xIC50	136	72
0.5xIC50	135	54
1xIC50	120	36
2xIC50	80	16
3xIC50	124	11

α = 1.0
Ki = 2.4 nM

Test condition	Substrate K_m (µM)	Substrate V_{max}
DMSO	113	139
0.25xIC50	64	69
0.5xIC50	46	44
1xIC50	26	25
2xIC50	23	17
3xIC50	15	11

α = 0.1
Ki = 26 nM

Figure 1: Examples of compounds with varying types of non-competitive behaviour. A) Compound A shows a competitive element with α=2.0. There is a curvilinear increase in K_m and decrease in V_{max} with increasing [I]. **B)** Compound B shows less mixed inhibition with α=1.0. There is a curvilinear decrease in V_{max} and little effect of K_m with increasing [I]. **C)** Compound C shows an un-competitive element with α=0.1. There is a curvilinear decrease in V_{max} and a downward trend in K_m

References: Evaluation of Enzyme Inhibitors in Drug Discovery: A guide for Medicinal Chemists and Pharmacologists (2nd ed), Robert Copeland, 2013

3 RESULTS (continued)

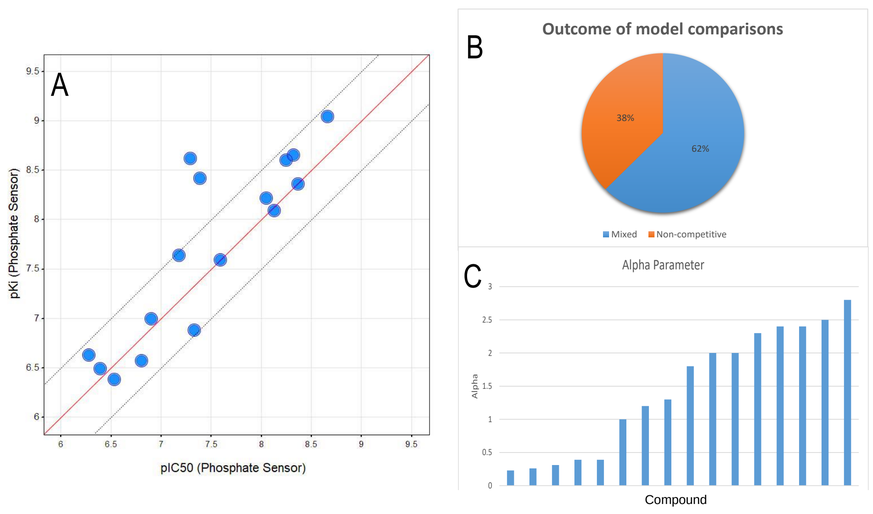


Figure 2: Summary of results of mechanistic analysis of 16 ATPase inhibitors. A) There is a good correlation between each compound's respective pIC50 and pKi, determined using the appropriate model in GraphPad Prism. **B)** The majority of the compounds tested show a mixed mechanism (62%). **C)** Alpha parameters for each compounds range from 0.1 to 2.8, demonstrating varying flavours of mixed inhibition

4 CONCLUSIONS

- In collaboration with our client we have established a capability that enables us to determine the modality of enzyme inhibitors
- The fluorometric phosphate sensor assay allowed us to kinetically profile sixteen ATPase inhibitors and distinguish between differing inhibition modalities with throughput being twenty compounds per day
- No compounds showed a pure competitive or uncompetitive mechanism; in 2/3 of cases compounds were found to be non-competitive with competitive or uncompetitive aspects to their modality, as demonstrated by their respective α parameters; these were reported as "mixed"
- All analysis was performed in GraphPad Prism which proved to be time intensive with data turnaround of around one day for four compounds
- Going forward the there are plans to invest in the Genedata MOA package with vastly decreased data turnaround time, for utilisation in future projects at Charles River Discovery