

Mechanism Of Action Study

Sample Project Study Report

Task Order: #DEMO

Applicable to all LabChip® assays, including:

- Protein kinases
- Lipid kinases
- Phosphatases
- Proteases
- Phosphodiesterases (PDEs)
- HistoneDeAcetylases (HDACs)

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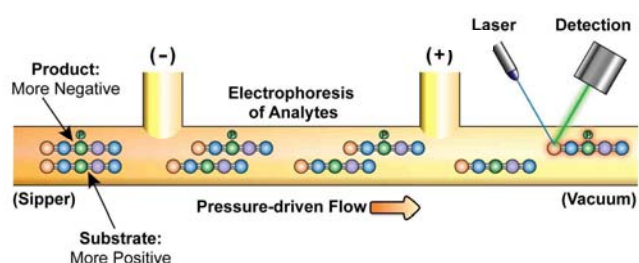
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Executive Summary of the Study

- This report is for demonstration purposes only. Actual results and experimental details will vary by the target assayed and by the inhibitor that is characterized.
- A potent inhibitor (ID = _____) was tested in dose response to establish its IC_{50} at the target of interest. An IC_{50} value of _____ was determined.
- The reversibility of inhibition was characterized.
- The compound was characterized for possible time-dependent inhibition.
- The mode of inhibition ie, competitive, non-competitive or uncompetitive was determined to be _____.
- An off-rate could not be determined because initial experiments indicate it was too fast to be measured.

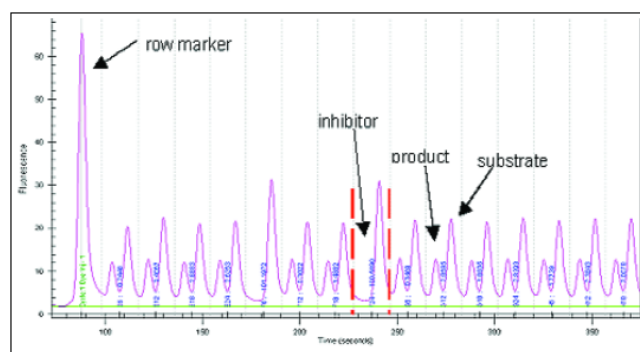
1. Background Information

This project was completed using the Caliper LC3000 Drug Discovery Platform and 12-sipper chips. LabChip® assays are separations-based, meaning that the product and substrate are electrophoretically separated, thereby minimizing interferences and yielding the highest data quality available on any screening platform. Z' factors for LC3000 enzymatic assays are routinely in the 0.8 to 0.9 range. High Z' values, few false positives, few false negatives and analytical quality reproducibility are the reasons cited for the increasing reliance on the LabChip® assays.



The off-chip incubation mobility-shift assay uses a microfluidic chip to measure the conversion of a fluorescent substrate to a fluorescent product. The reaction mixture, from a micro-titer plate well, is introduced through a capillary sipper onto the chip, where the substrate and product are separated by electrophoresis and detected via laser-induced fluorescence.

The signature of the fluorescence signal over time reveals the extent of the reaction.



Separation and detection of product and substrate allows accurate quantification of inhibition.

The precision of microfluidics allows researchers to detect subtle interactions between drug candidates and therapeutic targets. The technology is able to detect both strong and weak enzyme modulators with high accuracy, and routinely identifies drug candidates that conventional techniques miss.

2. IC₅₀ Determination

- An IC₅₀ value is determined in the standard assay protocol. This value is used in subsequent experiments,
- Ten concentrations of drug are tested in duplicate at a fixed time point and substrate concentration,
- 12 ul of enzyme (2.1X) are added to 1 ul of 25X test sample in 10% DMSO and allowed to incubate for 15 minutes before adding 12 ul of substrate (2.1X),
- RT incubation for 1 hour,
- Reaction stopped and read on LC3000,
- Analyze results and graph in GraphPad. Fit to non-linear regression with variable slope, constraints of 0 and 100 for bottom and top, respectively.

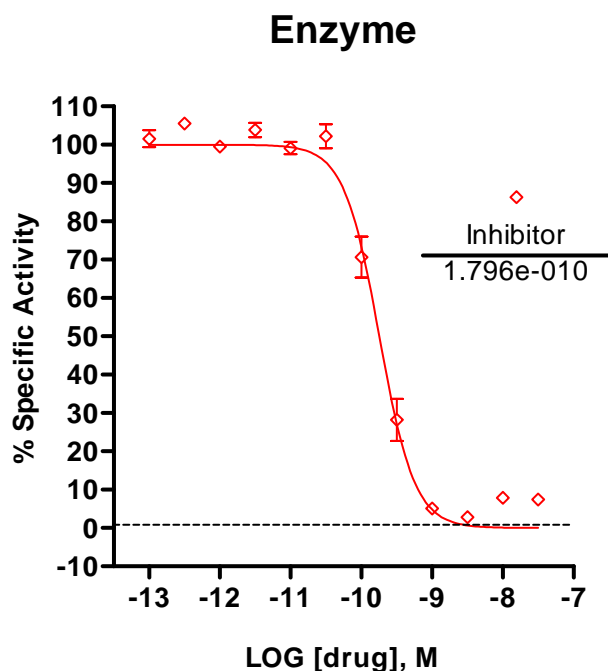


Figure 1: Inhibitor dose response in target enzyme. The IC₅₀ of about 0.2 nM is consistent with other values reported for this inhibitor/target pair.

3. Reversibility of Inhibition

- Set up duplicate reactions containing sample at 0, 10, 30 and 100X the IC₅₀ value with 100X enzyme concentration. Preincubate for 30 minutes at room temperature,
- Initiate reaction by addition of substrate in buffer so that the enzyme is diluted to 1X and sample is diluted to 0, 0.1, 0.3 and 1X the IC₅₀ value,
- Read kinetically,
- Graph percent conversion vs. time for each sample concentration,
- Analyze for linearity over time and determine reaction rates.

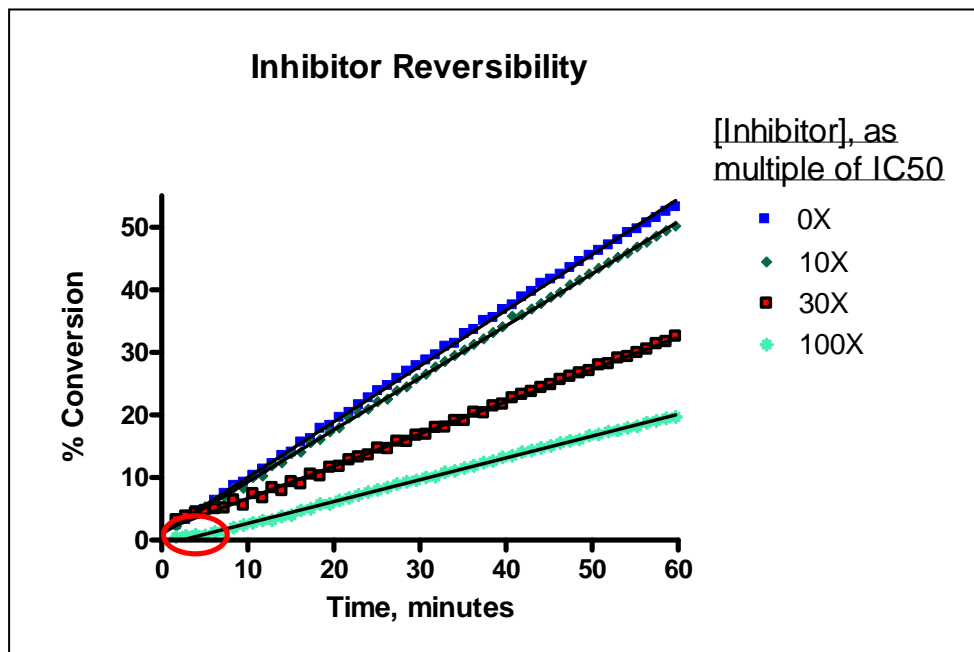


Figure 2: The inhibition of enzyme by this inhibitor satisfies our definition of reversibility (*conversion rate in 10X well >80% of uninhibited (0X) rate*). At higher sample concentrations, there appears to be some delay for dissociation (red circle). This is also shown in the reduced rate of conversion (39%) relative to the predicted rate (50%) for the 1X concentration at reaction.

[Sample] as IC50 Multiple in Preincubation	[Sample] as IC50 Multiple in Rxn.	% Conversion/min	Actual Conversion Rate as % of Control	Predicted Conversion Rate as % of Control	Variance
0	0	0.89	100	100	0
10	0.1	0.83	93	91	2
30	0.3	0.69	77	70	7
100	1	0.35	39	50	11

Table 1: Analysis of the reversibility of this inhibitor. At moderate and low concentrations there appears to be rapid and complete reversibility. At high concentrations, reversibility appears to be somewhat delayed.

4. Enzyme Linearity

- Setup duplicate wells with sample at 0, 0.25, 0.5, 1, 2, 4X IC₅₀ and substrate,
- Initiate reaction by addition of enzyme at 0.5X,
- Read kinetically,
- Graph percent conversion vs. time for each sample concentration,
- Analyze for linearity over time and determine reaction rates,
- Plot % conversion over time using GraphPad, check for linearity.

Inhibitor Linearity

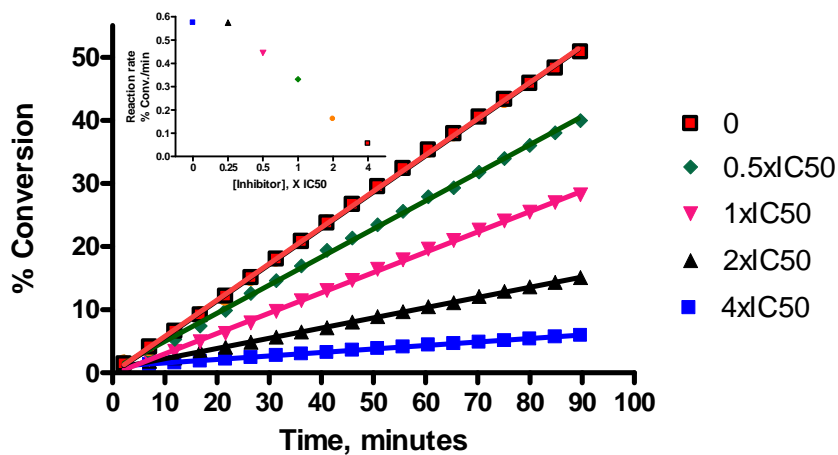


Figure 3: Enzyme activity is linear at all moderate concentrations of inhibitor. There is no apparent time-dependence for inhibition by this compound.

5. Determination of Mode of Inhibition

- Setup duplicate wells in a matrix of sample at 0, 0.25, 0.5, 1, 2, 4X IC_{50} and substrate at concentrations around substrate K_m ,
- Initiate reaction by addition of enzyme,
- Read kinetically on LabChip 3000,
- Graph conversion rates,
- Analyze in GraphPad; compare fit for competitive, noncompetitive and uncompetitive models.

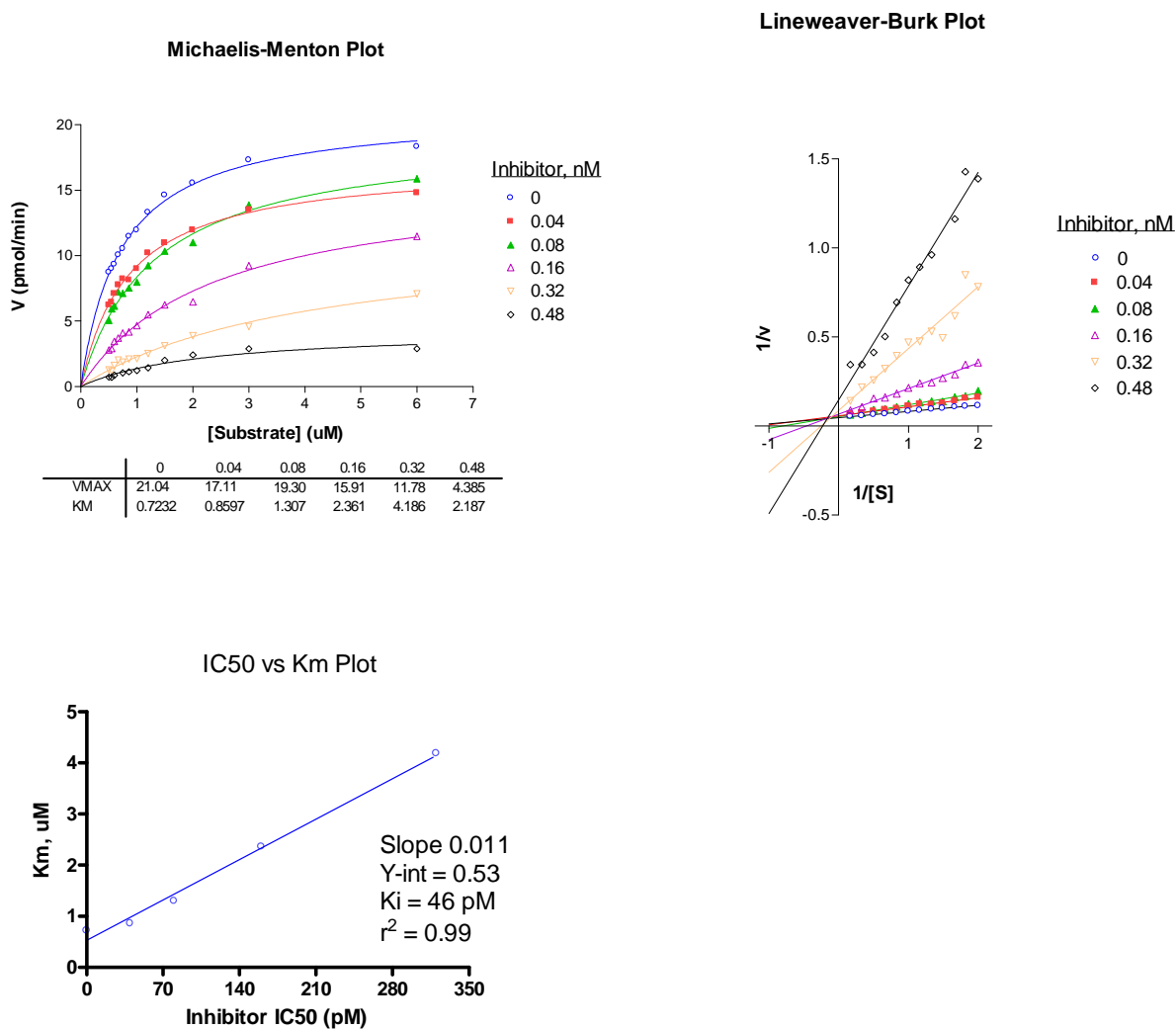


Figure 4: This compound inhibits the enzyme target in a competitive manner. The K_i value of about 50 pM is consistent with literature data, and with the K_i calculated using the Cheng-Prusoff equation from the IC_{50} determined in step 1.

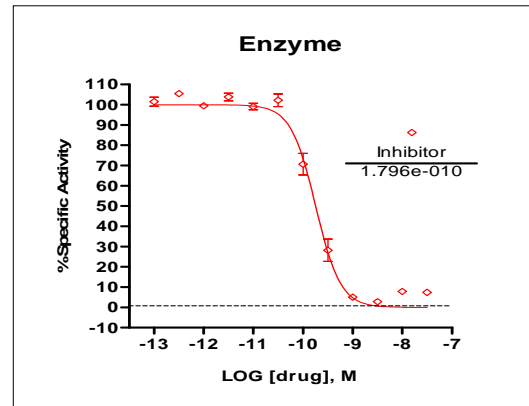
6. Koff Determination

- The off-rate for this inhibitor at this enzyme was not determined because previous data strongly indicated that the dissociation happens too quickly for measurement.

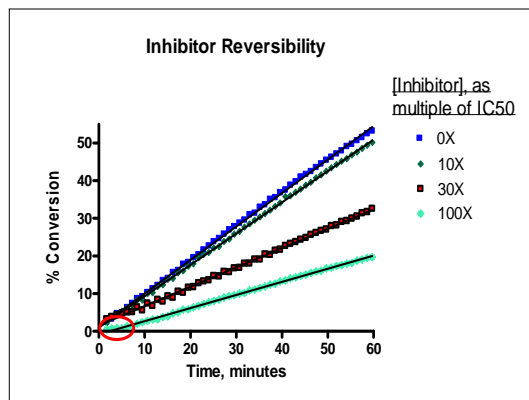
7. Reference

8. Technical Summary

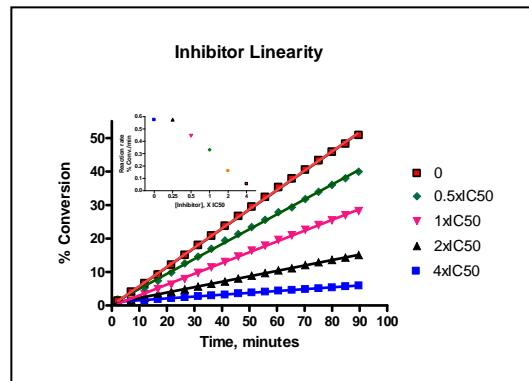
- An IC_{50} value of 0.18 nM was determined, and agrees with prior reported values. Inhibition was complete.



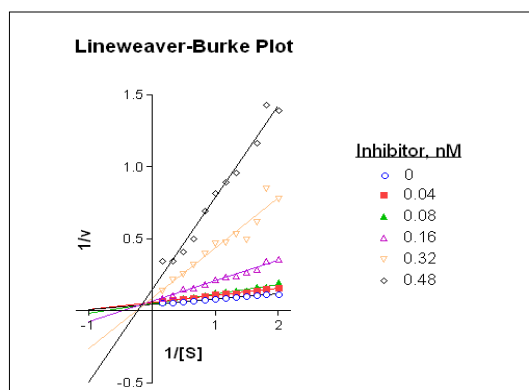
- Inhibition of enzyme activity is fully reversible. A short delay in the restoration of enzyme activity may have occurred with the highest inhibitor concentration tested, which is consistent with the variance in conversion seen.



- The sample showed no time-dependent inhibition at any concentration tested.



- The mode of inhibition was determined to be competitive with respect to substrate.



- A K-off experiment was not performed because earlier assays showed the off-rate was too fast to be measured.