Evaluating tight binding small molecule inhibitors using the Morrison equation

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INTRODUCTION

Generating accurate potency data is crucial for driving structure-activity relationship (SAR) work in drug discovery projects. In biochemical screens this is often focused on determining a compound IC_{50}, however K_i^{app} values are a more appropriate quantitative indicator of potency when looking at tight binding compounds, as well as more generally. A K_i^{app} provides the apparent dissociation constant of the enzyme-inhibitor complex (without mechanism of action corrections).

This project is focused on NAD$^+$-dependent EnzymeX. Current screening utilises the NADH-Glo Detection System from Promega (Figure 1), and publicly available tool compounds have been used as the starting point for chemistry.

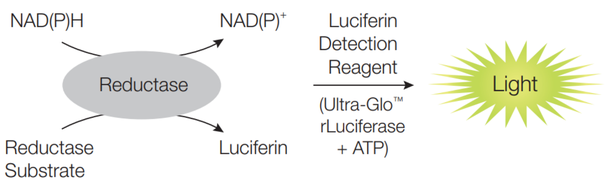


Figure 1: NAD(P)H-Glo Detection System schematic[1]. NADH levels can be quantified using the reductase, reductase substrate and luciferin detection reagent.

The tool compounds are highly potent and sit at the bottom of the assay – which is an issue when attempting to accurately measure potency. Initially to counteract this problem, the NADH-Glo assay was reoptimised to reduce the concentration of EnzymeX from 500 nM to 100 nM. The optimisation involved running the reaction at 37°C (Figure 2) and increasing reaction time. The concentration change was substantial; however, the most potent compounds were still at the limit of the assay. It was not possible to reduce the enzyme concentration any further whilst still generating robust data.

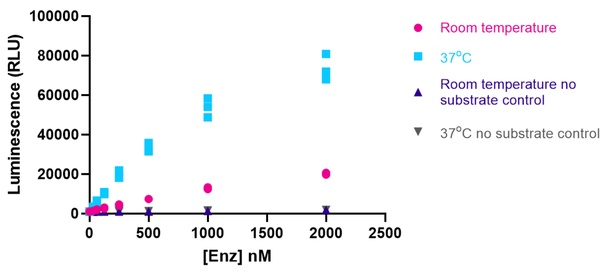


Figure 2: Raw luminescence at increasing EnzymeX concentrations, running reaction at room temperature and 37°C (with and without substrate control).

It was decided that it would be more beneficial to alter the analysis process rather than the assay format, to accommodate these tight binding compounds.

OBJECTIVE

Transfer from using routine IC_{50} data analysis to using the Morrison equation to generate K_i^{app} values and drive forward lead identification.

MORRISON EQUATION

The main difference between IC_{50} and Morrison analysis is that the concentration of free inhibitor is no longer assumed to be equal to the concentration of total inhibitor added to the system. This is important when studying tight binding compounds, where there is a depletion of free inhibitor with the formation of enzyme-inhibitor complexes.

Tight binding conditions have more of an effect as the IC_{50} approaches the enzyme concentration, and the analysis limit is found where $IC_{50} < ½[E]$. Under these conditions, compound potencies can be hard to differentiate as they tend to a similar IC_{50} value. By switching to the Morrison equation (see Figure 3), we hope to improve upon this threshold. The hypothesised lower limit of the Morrison equation is $K_i^{app} < 1/100[E]$ and this should allow for better ranking of the most potent compounds being synthesised.

$$\frac{v_i}{v_0} = 1 - \frac{([E]_T + [I]_T + K_i^{app}) - \sqrt{([E]_T + [I]_T + K_i^{app})^2 - 4[E]_T[I]_T}}{2[E]_T}$$

Figure 3: Morrison equation[2]: where v_i is measured velocity; v_0 is velocity in the absence of inhibitor; $[E]_T$ is total enzyme concentration; $[I]_T$ is total inhibitor concentration.

RESULTS

For the best results, the total enzyme concentration ($[E]_T$) term was constrained in the Morrison equation – requiring the determination of the enzyme active site concentration. Generally, enzymes are not 100% pure or active, so an active site titration was completed.

EnzymeX was titrated in the presence of an excess of tight binding compound (200x K_i), providing a value of 1954 nM (n=1) from the x-axis intercept (Figure 4). The n=2 average of the intercept was then converted to give 0.094 µM active sites from the 100 nM enzyme screening concentration.

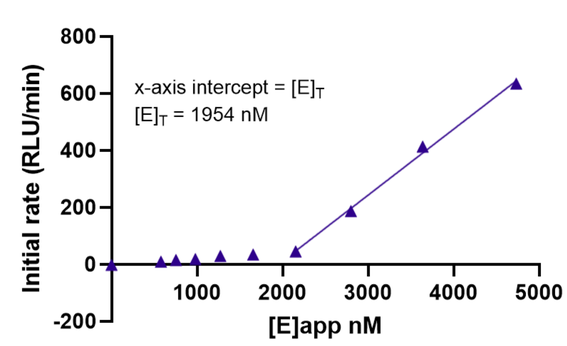


Figure 4: EnzymeX titration in the presence of 2 µM tight binder. X-axis intercept at 1954 nM .

In way of testing the assay limit hypothesis, the same concentration response data was analysed in ActvityBase to generate IC_{50} values and in Prism to generate K_i^{app} data. Comparing pIC_{50} and pK_i^{app} values (Figure 5) shows that it has been possible to move through the lower limit of the NADH-Glo assay. The data correlates well when looking at compounds between 4.5 – 6.5 pIC_{50}, however above that, the pK_i^{app} values shift away from the y=x correlation. Many of these have potency shifts greater than 0.25 log. IC_{50} measurements underestimate the true potency of these tight binding compounds.

A vital next step after biochemical screening, is being able to transfer this inhibitory effect into a cellular environment. A selection of compounds were run in an in-cell ELISA biomarker assay to assess activity (Figure 6). The plot shows that the compounds appear to be inhibiting EnzymeX in in a cellular context: with a better correlation between biomarker and pK_i^{app} values, opposed to pIC_{50} values. There is a general shift in compound potency in cells compared to the biochemical data, but this is often expected. However, the correlation provides evidence of the translatability of compounds between these two SAR assays.

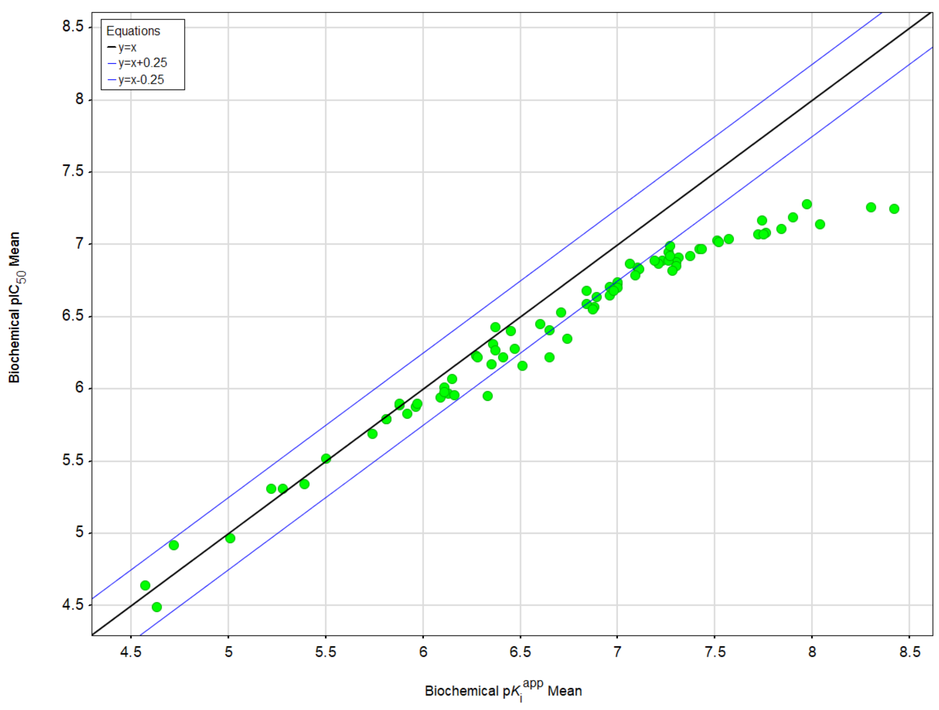


Figure 5: Correlation plot of average pIC_{50} and pK_i^{app} data from compounds tested in the NADH-Glo Enzyme X screening assay.

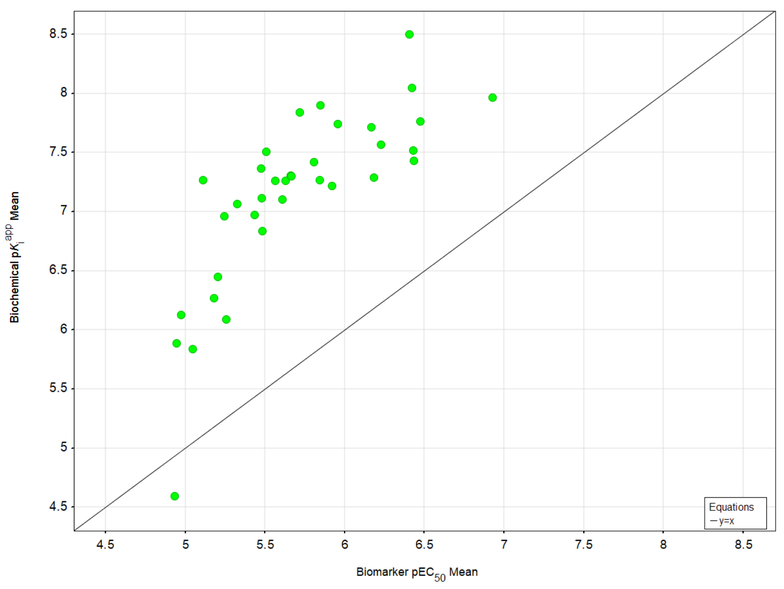
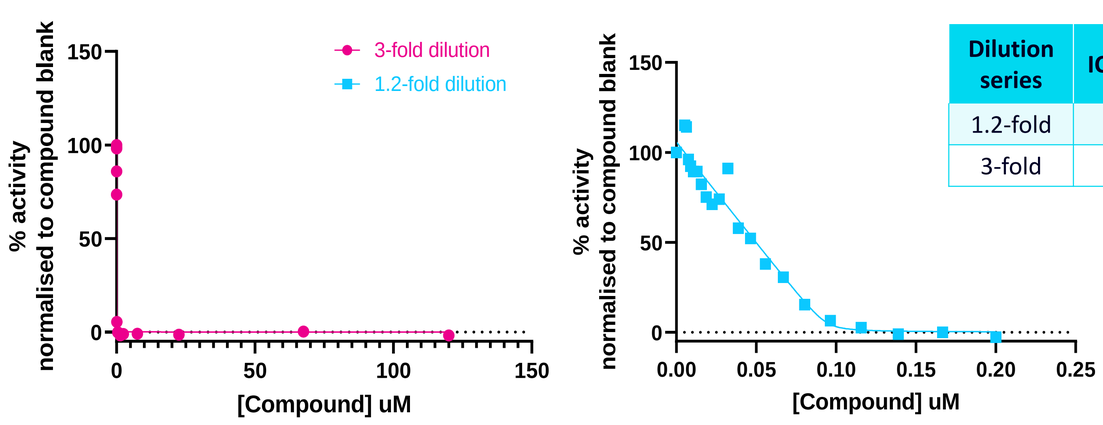


Figure 6: Correlation plot of average pK_i^{app} (NADH-Glo biochemical assay) and biomarker pEC_{50} data (in-cell ELISA assay).

Whilst running SAR, we came across several very potent compounds which were not well characterised by the 3-fold, 12-point compound dilution series. Based on our data, the limit of the assay was ~50-fold below the [E]. Therefore, the screening format required further testing to produce accurate data for these compounds of interest. Further optimisation was completed in terms of altering the dilution series used to test compounds. Copeland[3] suggests an optimal fold dilution of 1.5, and this was done to increase the number of points in the crucial elbow section of the concentration response curves.

Experiments were completed to assess if a tighter dilution series would affect K_i^{app} values. There was not a significant change in the IC_{50} values generated from the data (Figure 7), whereas the K_i^{app} was shifted to a higher potency with the increased number and better distributed range of points.



Dilution series	IC_{50} (nM)	K_i^{app} (nM)
1.2-fold	53.4	0.270
3-fold	56.6	5.74

Figure 7: EnzymeX % activity graphs showing activity of a tight binding compound. Left-hand graph shows 3-fold, 12-point data and the right-hand graph shows 1.2-fold, 16-point data. Corresponding IC_{50} and K_i^{app} values are recorded in the table.

CONCLUSION

It has been possible to circumvent the issue of high assay enzyme concentration and tight binding compounds. SAR screening has been transferred from 3-fold to 1.5-fold compound dilutions, to improve the spread of data points in analysis. All data is now analysed using the Morrison equation and has provided project chemists with more accurate potency data for progressing inhibitory compounds.

Promising biomarker data has increased confidence in the translatability of project compounds from biochemical to cellular assays. Future work includes automating the Morrison equation analysis process in ActivityBase to improve the efficiency of the SAR cycle and ensure data integrity. Setting up this analysis protocol will help future projects that may benefit from generating K_i^{app} values.

REFERENCES

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[2] Rodrigues, M. V. N., Corrêa, R. S., Vanzolini, K. L., Santos, D. S., Batista, A. A., Cass, Q. B., 2015. Characterisation and screening of tight binding inhibitors of xanthine oxidase: and on-flow assay. *RSC Advances, 47*

[3] Copeland, R. (2013) *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists.* 2nd edn. John Wiley & Sons.