Adjusting the fitting of fluorescence-based dose response kinase inhibition assay to account for fluorescent inhibitors

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Authorship contribution statement
GP is responsible for all the work acquired and presented in this manuscript: conceptualization, data acquisition, data curation and formal data analysis, writing and editing of the original manuscript and preparation of figures and tables.

1 Abstract

Fluorescence is routinely used to monitor kinase inhibition in commercial assays. Occasionally fluorescent compounds can interfere with the fluorescent reading. To address this issue, the problematic data is usually truncated to improve the fit, however this approach raises ethical and reproducibility concerns. Instead, it is suggested to adjust the fitting formula (figure 1), to account for the autofluorescence of the compounds and improve the fit of the data compared to a naïve approach. Finally, it was noticed that truncating the data can results in small underestimation of the IC_{50} values and should therefore be used carefully.

Keywords: Kinase binding, LanthaScreen, Fluorescent compound, Autofluorescence, small molecule kinase inhibitor.

2 Introduction

Since the early 2000s, kinase inhibition has been regarded as a successful strategy to tackle cancers inter alia, resulting in over 100 such drugs approved by different organisations in 2023. The majority of kinase inhibitors are small molecules (small molecule kinase inhibitors, SKMI) [1,2] and in parallel to their development, assays to measure kinase inhibition have also appeared and improved rapidly.[3] A popular option to test potential kinase inhibitor is Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET) that assesses binding of compounds to kinases active sites.[4,5] In these assays, the kinase of interest is labelled with lanthanide tagged antibodies, with affinity for the kinase purification tag, while tracers linked to fluorophores bind the kinase active site (Figure 2A). The TR-FRET signal between the antibody and the tracer is then monitored with a fluorescence plate reader. In a typical experiment, the tracer must compete with the SMKIs for the ATP binding pocket, which leads to a reduction of the TR-FRET signal based on affinity and concentration of the SMKIs (Figure 2B). Finally, the measured fluorescent signal intensity is plotted against the compound concentration and these values are fitted with Equation 1 to determine the IC_{50} values of each SMKI.

$$Y = \ bot + \frac{(top - bot)}{1 + 10^{(X-K)}} \qquad \text{Equation 1}$$

Where Y is the fluorescent intensity, X the Log_{10} of the compound concentration and K is the $Log_{10}(IC_{50})$.

Equation 1 can be derived from Hill equation [6] which, despite its simplicity, is still in use today (also described as “Hill-Langmuir equation”).[7] Equation 1 reports on the complex formation indirectly hence allowing for parameters such as *bot* and *top* which account for the background signal and signal amplitude. Additionally, Equation 1 takes concentrations in a logarithmic scale, which is practical for experimental set-ups where compounds are prepared through dilution series. However, experiments are usually imperfect, and the measured fluorescent signal Y describes more than the fraction of complex formed. Any contribution to the fluorescence is included in Y, including phenomenon such as the autofluorescence of the components tested (Figure 2C). When these external sources of fluorescence become dominant, the fitting of the data does not reliably represent the IC_{50} values anymore and the model needs to be adjusted. The current recommendation to treat datapoints that drift from the *bot* parameter, due to fluorescent compounds for example, is to ignore these points. This approach allows for a quick and easy fix which does improve the fitting and can sometimes help calculate IC_{50} values even when the data suffers from drifting. However, removing datapoints arbitrarily raises reproducibility issues (are the removed datapoints always mentioned in the methods?) and ethical ones (where is the line between removing an outlier and removing points to make the data fit better to the initial hypothesis?). Here, I propose a simple adjustment to Equation 1, to account for compound autofluorescence instead. This adjustment improves the results compared to a naïve fit, without having to (arbitrarily) truncate the data either.

3 Results

3.1 *Treating the autofluorescence of compound as linear*

It was assumed that, at low species concentration, everything else being equal, the fluorescence of the species increases linearly with its concentration.[8] This assumption does not apply to phenomena such as J-aggregation or other types of aggregation that affect the fluorescence in non-linear fashion.[9] However, it has the merit of simplicity and is consistent with the observation that the fluorescence of the compounds presented in this manuscript increased linearly with their concentration in aqueous solution and in absence of tracer or europium-tagged antibodies. Therefore, the autofluorescence of the compound was approximated with the equation: $Y = \ m \times L$ where Y represents the fluorescent reading value, L the concentration of the species and m is a parameter which englobes all the other factors (large m correspond to highly fluorescent compounds whereas a m close to 0, mean that the compound does not fluoresce).

After converting it to the logarithmic form, the linear equation was combined with Equation 1 resulting in Equation 2.

$$Y = \ bot + \frac{(top - bot)}{1 + 10^{(X-K)}} + m \times 10^X \qquad \text{Equation 2}$$

Where Y is the fluorescent reading, K is the $\mathrm{Log}_{10}(\mathrm{IC}_{50})$, X is the Log_{10}(Concentration) and *top*, *bot* and m are parameters governed by the experimental set up.

3.2 *Experimental testing of Equation 2*

~~Find the method and raw data in the supplementary material~~.

Five datasets with varying signal amplitudes and signal-to-noise ratio were selected to test and assess Equation 2. These datasets are representative of many situations encountered while performing LanthaScreen™ kinase binding assays. The raw data is available in the supplementary material.

Dataset 1 shows “good” data: high signal-to-noise ration and little sign of autofluorescence.

In datasets 2 and 3, the signal is not as good, the emission ratio (the emission ratio is proportional to the TR-FRET signal intensity) amplitude is much smaller, in these cases the autofluorescence of the compound becomes noticeable, there is no obvious plateau of the emission ratio at high compound concentration that can be described by the parameter *bot*. Dataset 4 is an extreme such case; and the typical recommendation here is to truncate the last 2-3 data points to improve the fit. Finally, dataset 5 comes from data with very small amplitude (kinases or antibodies are degraded), in this case the signal-to-noise ratio is small and the autofluorescence of the compound is distorting the results. This data should typically be reacquired if possible. To compare Equation 2 with the current recommendation, the datasets were analysed with three different approaches:

1) A naïve fit approach, simply fitting the data with Equation 1 without additional curation.

2) A truncated fit approach: upon inspection of the data, the points that deviate from the fit with Equation 1 were removed and the truncated data was fitted once again with Equation 1.

3) An adjusted fit approach: fitting the data as is, with an equation that accounts for the fluorescence of the compound (Equation 2) keeping all the datapoints in.

In dataset 1, where the data is considered “good”, the results are the same regardless of the method used for the fitting (which is good! See Table 1).

In the second example presented, datasets 2 and 3, the deviation from a flat *bot* plateau, caused by the fluorescence of the compounds is more marked than in dataset 1. Obviously the “naïve” approach should be avoided upon inspection of the data (Table 2).
On the other hand, the truncated fit approach, which omits the problematic points (the one that have a clear deviation from the sigmoidal fit) leads to improved R^2 values and more realistic results. Similarly, the adjusted fit approach also leads to improved results compared to a naïve fit but, in addition, considers all the data, and avoids (arbitrarily) omitting “outliers” (Table 2).

In the lasts two examples (datasets 4 and 5, Table 3), where the data is heavily affected by the autofluorescence of the compounds, the naïve approach completely breaks down. In dataset 4, removing the points with high autofluorescence can be justified since most of the data seems to actually follow the sigmoidal fit (Table 3). In dataset 5, the data is very noisy. To improve the fit, one needs to remove many datapoints, which leaves barely enough for fitting (Table 3). Moreover, the IC_{50} value varies depending on which data points are kept or removed. If the data cannot be reacquired, the adjusted fit methods should be preferred.

3.3 Truncation of the data can lead to systematic underestimation of IC_{50} values.

It was noticed that the IC_{50} value is always smaller for the truncated fit compared to the adjusted fit approach. In equation 1, the values for the parameter *bot* should coincide with the plateauing of emission ratio values at high compound concentration. However, for the adjusted fit, the fluorescence values measured are the sum of the autofluorescence of the compound ($Y = m \times 10^X$) and the FRET signal between the Eu-tagged antibody and the tracer, ($Y = bot + \frac{(top-bot)}{1+10^{(X-K)}}$, see Figure 1). At low compound concentration, the contribution from $Y = m \times 10^X$ is very small, but as the compound concentration increases so does the autofluorescence of the compounds. As a result, the effective *bot* parameter should be smaller than when ignoring the autofluorescence of the compounds. Smaller *bot* parameters lead to larger IC_{50} values compared to the naïve or truncated fit (Figure 1).
Based on the adjusted fit model, it appears that truncating data deviating from the fit with Equation 1, leads to underestimation of the IC_{50} value.

4 Conclusion

In the context of data fitting in a dose-response experiment, this manuscript describes a simple yet effective adjustment of the typical fit with Equation 1, to include the contribution from the autofluorescence of compounds. Of importance, this equation assumes a linear increase of the fluorescence with the compound concentration, which is well suited to water-soluble compounds, but has not been tested to describe the fluorescence drift linked to protein-compound aggregation or J-aggregation, sometimes affecting fluorescence-based assays.[9]

When a good signal is measured and compounds tested are not fluorescent, the classical naïve approach is perfectly fine (for example dataset 1) and should be preferred because of its simplicity. However, when working with fluorescent compounds, the naïve approach does not describe the data adequately anymore and it becomes necessary to adapt the fitting method. Traditionally, outliers were removed until fitting improved, and while this approach can be legitimate in some situation (dataset 4 for example) it can also lead to reproducibility issues. It was also shown that truncating data can lead to underestimation of the IC_{50} values (Figure 1) by neglecting the contribution of the compound fluorescence to the parameter *bot* at higher compound concentrations. For this reason, I believe that the adjusted fit (Equation 2) should be prefer over omitting data.

Natural science is facing a “reproducibility crisis” and among the many potential reasons specified, one find selective reporting, poor analysis and unavailability of raw data.[10] I hope that Equation 2 can simplify some of the analysis with fluorescent compounds. This adjusted approach is not a substitution for inspecting the data carefully, especially in situations where they behave in an unexpected manner. In the end, it will be up to the experimenters to find the balance between simplicity and correctness, to determine whether their data is good enough to be fitted with the naïve approach, or if Equation 2 is better suited instead.

To conclude I would like to share the idea from George Box that: “All models are wrong, but some are useful”.[11]

5 Acknowledgments

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5.1 Competing interests

The author has no relevant financial or non-financial interest to disclose.

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Figure 1

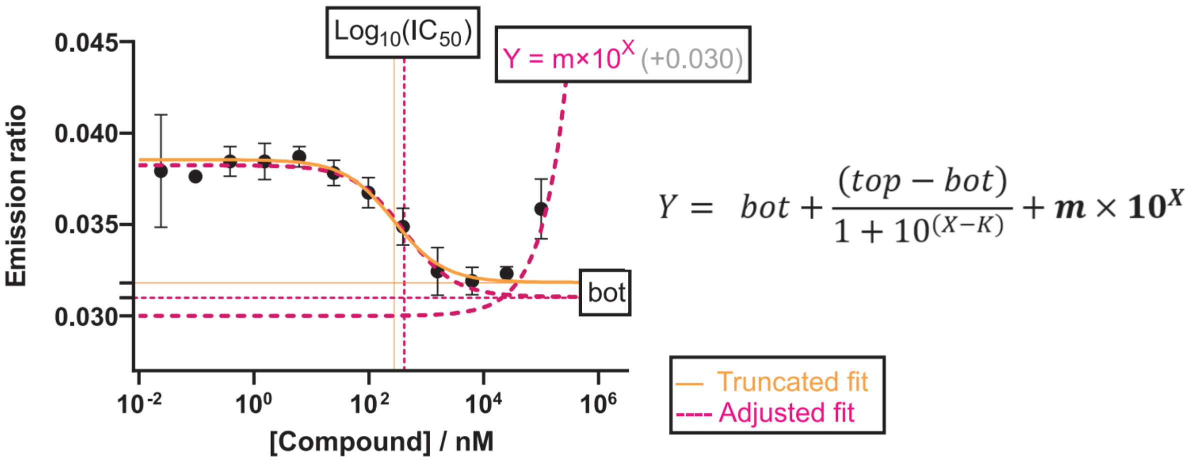

Figure 1: Accounting for autofluorescence of compounds in fluorescent based kinase binding assays. On the left panel, fluorescence intensity (emission ratio) from LanthaScreen ™ kinase binding assay are plotted against compound concentration (logarithmic scale) and fitted with two different approaches. First, a truncated fit (orange), where the two last points at highest compounds concentration have been omitted for the fitting, as they do not follow the sigmoidal curve. In the second approach, the fitting method is adjusted by adding the term $m\times10^X$ to the typical logistic function, to account for the fluorescence of the compounds (equation on the right). The adjusted approach does not require trimming the data and results in having slightly lower values for the parameter bot, resulting in slightly larger IC_{50} values. Y is the fluorescent signal, top and bot are parameters representing the minimum and maximum signal. X is the logarithmic concentration of the compound and K is the $Log_{10}(IC_{50})$ value of the reaction.

Figure 2

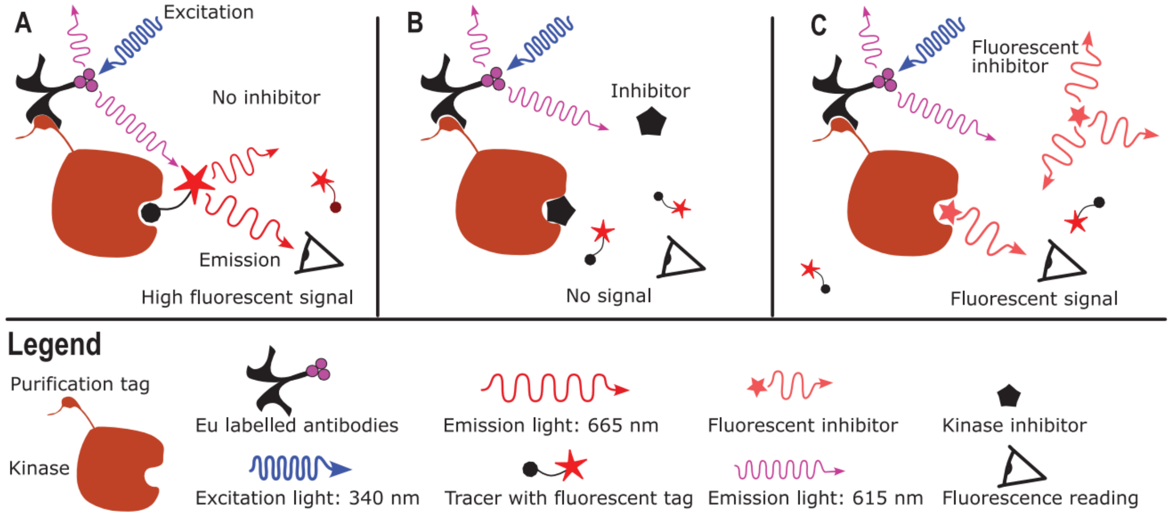

Figure 2: Graphical summary of TR-FRET kinase binding assay. (A) A system made of Europium labelled antibody, kinase and fluorescent tracer, relays a signal that can be monitored at wavelength 665 nm with a fluorescent plate reader. (B) When adding inhibitor to the system, the tracer has to compete with the inhibitor for the kinase active site and the signal intensity is reduced proportionally to the binding of the inhibitor to the kinase. (C) Occasionally the inhibitor itself can be fluorescent, in which case the signal may increase proportionally to the inhibitor concentration in addition to the fluorescence resulting from the europium-tracer relay.

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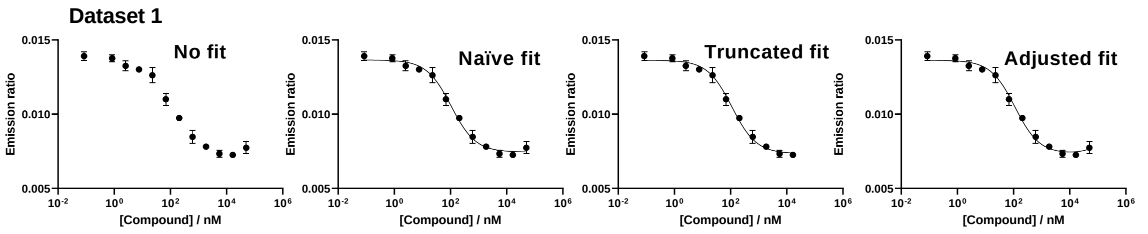
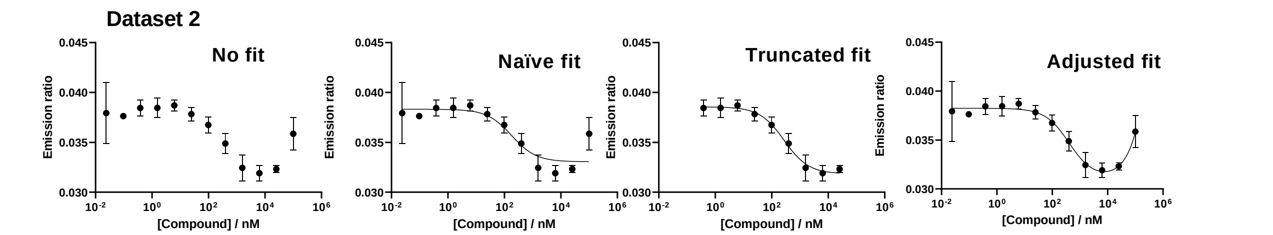
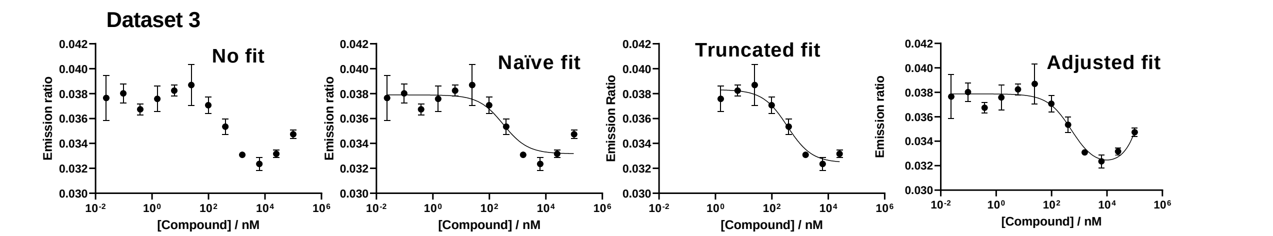


Fit	IC_{50} (nM)	Bot	Top	m	#Data	R^2
Naïve	**105.2** (83.5 to 132.6)	**0.0074** (0.0072 to 0.0077)	**0.0137** (0.0134 to 0.0139)	-	36/36	0.985
Truncated	**111.5** (88.5 to 140.5)	**0.0073** (0.0071 to 0.0076)	**0.0136** (0.0134 to 0.0138)	-	33/36	0.986
Adjusted	**112.8** (87.3 to 145.6)	**0.0073** (0.0070 to 0.0076)	**0.0136** (0.0134 to 0.0139)	**$6.59e^{-9}$** ($-4.038e^{-9}$ to $1.733e^{-8}$)	36/36	0.985

Table 1: Fitting good quality data. Top panel represent Dataset 1 fitted with the three different approaches: naïve, truncated and adjusted. The Y axis shows the emission ratio and the X-axis the compound concentration on a logarithmic scale. The bottom panel summarises the parameters from the different fitting approaches, with the best fit value in bold and the 95% CI for the value given in parentheses. # Data represent the number of points used for the fit compared to the number of point available, R^2 represents the non-linear goodness of the fit using the different approaches.

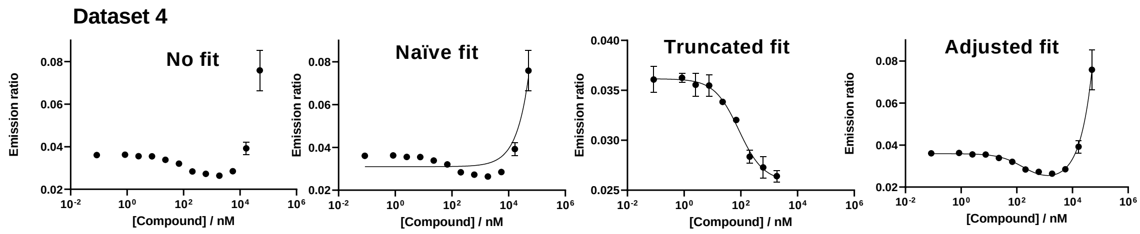


Fit	IC_{50} (nM)	Bot	Top	m	#Data	R^2
Naïve	**179.8** (60.2 to 497.3)	**0.0331** (0.0321 to 0.0340)	**0.0373** (0.0376 to 0.0391)	-	36/36	0.710
Truncated	**274.9** (156.5 to 476.1)	**0.0318** (0.0312 to 0.0325)	**0.0386** (0.0381 to 0.0391)	-	27/36	0.931
Adjusted	**416.3** (190.2 to 895.0)	**0.0310** (0.0297 to 0.0322)	**0.0382** (0.0377 to 0.0388)	**$4.74e^{-8}$** ($2.846e^{-8}$ to $6.713e^{-8}$)	36/36	0.844

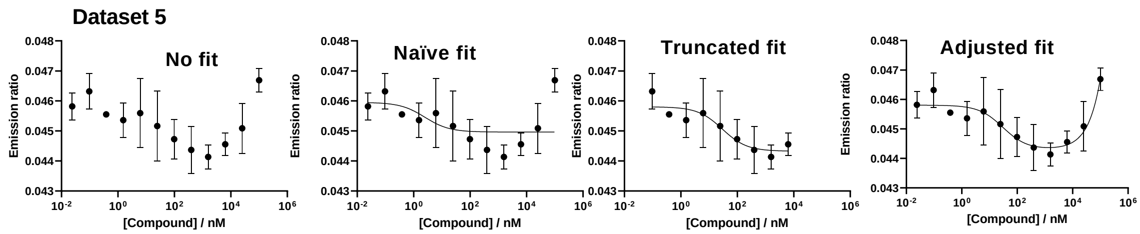


Fit	IC_{50} (nM)	Bot	Top	m	#Data	R^2
Naïve	**311.6** (137.8 to 689.5)	**0.0332** (0.0324 to 0.0339)	**0.0379** (0.0374 to 0.0384)	-	36/36	0.779
Truncated	**354.3** (180.0 to 694.9)	**0.0325** (0.03167 to 0.03318)	**0.0383** (0.0377 to 0.0390)	-	24/36	0.891
Adjusted	**533.2** (262.7 to 1079)	**0.0319** (0.0308 to 0.0329)	**0.0379** (0.0374 to 0.0383)	**$2.90e^{-8}$** ($1.315e^{-8}$ to $4.557e^{-8}$)	36/36	0.847

Table 2: Datasets 2 and 3 were fitted with the three different approaches: naïve, truncated and adjusted. For the truncated fit approach, outliers both at high compound concentration and noisy data at low compound concentration were removed (for a total of 12 points removed in dataset 2 and 15 points in dataset 3). In this example, the naïve fit gives poor results, underestimating the IC_{50} values as the measured fluorescence of points at high compound concentration is increasing again. Unsurprisingly, R^2 values are also worse for the naïve fit approaches. The R^2 values for the adjusted fit are not as good as that of the truncated fit, but mainly because of keeping the points with high standard deviation at low compound concentration. The 95% CI interval for the IC_{50} value are larger for the adjusted fit, which is made even more apparent by to the logarithmic nature of the X-axis.



Fit	IC_{50} (nM)	Bot	Top	m	#Data	R^2
Naïve	**~1.9×10^8** (NA)	**160** (NA)	**0.0310** (0.0290 to 0.0330)	-	36/36	0.832
Truncated	**85.5** (58.46 to 125.0)	**0.0259** (0.02497 to 0.02671)	**0.0362** (0.0357 to 0.0367)	-	27/36	0.962
Adjusted	**174.5** (62.4 to 504.4)	**0.0226** (0.01957 to 0.02503)	**0.0359** (0.0343 to 0.0376)	**1.06e^{-6}** (9.693e^{-7} to 1.150e^{-6})	36/36	0.962



Fit	IC_{50} (nM)	Bot	Top	m	#Data	R^2
Naïve	**2.50** (0.00 to 194.5)	**0.0450** (0.0445 to 0.0454)	**0.0460** (0.0453 to 0.0518)	-	36/36	0.178
Truncated	**29.4** (0.72 to 297.1)	**0.0443** (0.04375 to 0.04482)	**0.0458** (0.0453 to 0.0465)	-	27/36	0.474
Adjusted	**30.4** (2.39 to 219.1)	**0.0443** (0.0438 to 0.0447)	**0.0458** (0.0454 to 0.0462)	**2.41e^{-8}** (1.505e^{-8} to 3.339e^{-8})	36/36	0.576

Table 3: Fitting problematic datasets. The three different fitting approaches to datasets 4 and 5 lead to largely different results. The naïve fit should be avoided as it fails to generate any useful results, even if the R^2 value appears to be reasonable in the case of dataset 4 (which stresses that looking at the R^2 alone is not enough to determine whether a fitting approach is suitable!). Dataset 5 shows very noisy data, where the emission ratio range is quite small due to problems with the experimental set up. In this case it is not so clear which data points should be truncated and the IC_{50} value changes depending on which data is omitted, making this approach problematic. On the other hand, with the adjusted fit, truncating data is not necessary, although the data should be reacquired to improve the confidence in the results. Note that the range of the emission ratio for dataset 4, truncated fit, has been "zoomed in" compared to the two other approaches, to improve clarity.