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# Appendix 1

## Derivation of the Equilibrium Binding Dissociation constant, $K_d$

In order to define $K_d$, it is necessary to consider the equations below. Consider a ligand binding event between a protein P, and a ligand L, to form the 1:1 complex PL as described by the following equilibrium:
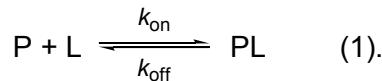
$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \qquad (1).$$

Thus, the equilibrium binding association constant $K_a$ is then defined as:

$$K_a = [PL]/[P].[L] \approx k_{on}/k_{off} \qquad (2).$$

Then, the equilibrium binding dissociation constant, $K_d$ is defined as:

$$K_d = [P].[L]/[PL] \approx k_{off}/k_{on} = 1/K_a \qquad (3).$$

Now let

$$P_0 = \text{the total concentration of protein} = [P] + [PL] \qquad (4),$$
$$\text{and } L_0 = \text{the total concentration of ligand} = [L] + [PL] \qquad (5),$$

then $K_d$ may also be expressed as:

$$K_d = (P_0 - [PL]).(L_0 - [PL])/[PL] \qquad (6),$$

and [PL] is given by,

$$[PL] = \tfrac{1}{2}.\{L_0 + P_0 + K_d - [(L_0 + P_0 + K_d)^2 - 4L_0P_0]^{1/2}\} \qquad (7).$$

In practice, the $K_d$ is often referred to as [L] corresponding to 50% occupancy of the protein P with the ligand L. Thus, according to equation (3), if there is 50% occupancy of P, then [PL] = [P], and therefore:

$$K_d = [L] \qquad (8).$$

However, it is important to note that this [L] here refers to the 'residual', unbound ligand L, according to the equation

$$[L] = L_0 - [PL] \qquad (9).$$

**Table A-1:** NMR [$^1$H,$^{15}$N] HSQC chemical shift data for binding of **2** to Tec SH3 domain.

**d $^1$H (H-N) (ppm)**

| [2] (mM) | D196 | E193 | G226 | L197 | Q190 | S230 | T192 | W215 | W215e1 | W216 | Y227 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 7.61 | 8.18 | 8.55 | 8.73 | 8.22 | 7.96 | 8.88 | 8.48 | 9.99 | 8.98 | 9.23 |
| 62.5 | 7.64 | 8.22 | 8.59 | 8.90 | 8.18 | 7.86 | 8.81 | 8.35 | 9.79 | 8.95 | 9.31 |
| 125 | 7.67 | 8.24 | 8.61 | 8.99 | 8.16 | 7.81 | 8.76 | 8.29 | 9.69 | 8.91 | 9.35 |
| 187.5 | 7.68 | 8.26 | 8.62 | 9.04 | 8.15 | 7.78 | 8.73 | 8.26 | 9.63 | 8.89 | 9.38 |
| 250 | 7.68 | 8.26 | 8.62 | 9.06 | 8.14 | 7.76 | 8.71 | 8.23 | 9.60 | 8.88 | 9.39 |
| 625 | 7.70 | 8.28 | 8.65 | 9.14 | 8.12 | 7.72 | 8.67 | 8.18 | 9.51 | 8.87 | 9.43 |
| 1250 | 7.71 | 8.28 | 8.65 | 9.17 | 8.11 | 7.71 | 8.65 | 8.15 | 9.47 | 8.85 | 9.44 |

**Dd $^1$H (H-N) (ppm)**

| [2] (mM) | D196 | E193 | G226 | L197 | Q190 | S230 | T192 | W215 | W215e1 | W216 | Y227 | Mean | Std.Dev |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | | |
| 62.5 | 0.03 | 0.04 | 0.04 | 0.17 | -0.04 | -0.10 | -0.08 | -0.13 | -0.20 | -0.03 | 0.08 | | |
| 125 | 0.05 | 0.07 | 0.06 | 0.25 | -0.06 | -0.15 | -0.12 | -0.19 | -0.30 | -0.07 | 0.12 | | |
| 187.5 | 0.06 | 0.08 | 0.07 | 0.31 | -0.07 | -0.18 | -0.15 | -0.23 | -0.36 | -0.09 | 0.15 | | |
| 250 | 0.07 | 0.09 | 0.08 | 0.33 | -0.08 | -0.20 | -0.17 | -0.26 | -0.40 | -0.10 | 0.16 | | |
| 625 | 0.09 | 0.10 | 0.10 | 0.40 | -0.10 | -0.24 | -0.21 | -0.31 | -0.49 | -0.12 | 0.19 | | |
| 1250 | 0.10 | 0.11 | 0.10 | 0.43 | -0.11 | -0.26 | -0.23 | -0.33 | -0.53 | -0.14 | 0.21 | Mean | Std.Dev |
| Calculated $K_d$ (mM) | 163.2 | 97.57 | 108.9 | 105.3 | 129.6 | 110.4 | 135.0 | 108.8 | 116.4 | 168.4 | 108.1 | 122.88 | 23.77 |

**|Dd/Dd$_{max}$|**

| [2] (mM) | D196 | E193 | G226 | L197 | Q190 | S230 | T192 | W215 | W215e1 | W216 | Y227 | Mean | Std.Dev |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 62.5 | 0.31 | 0.42 | 0.39 | 0.39 | 0.38 | 0.40 | 0.33 | 0.40 | 0.38 | 0.25 | 0.38 | 0.37 | 0.05 |
| 125 | 0.54 | 0.62 | 0.61 | 0.58 | 0.58 | 0.59 | 0.53 | 0.58 | 0.58 | 0.51 | 0.57 | 0.57 | 0.03 |
| 187.5 | 0.66 | 0.76 | 0.71 | 0.70 | 0.70 | 0.70 | 0.63 | 0.68 | 0.68 | 0.66 | 0.70 | 0.69 | 0.03 |
| 250 | 0.70 | 0.81 | 0.76 | 0.76 | 0.76 | 0.79 | 0.72 | 0.77 | 0.75 | 0.74 | 0.75 | 0.76 | 0.03 |
| 625 | 0.90 | 0.93 | 0.98 | 0.93 | 0.90 | 0.94 | 0.90 | 0.92 | 0.93 | 0.85 | 0.92 | 0.92 | 0.03 |
| 1250 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |

Table A-1 above contains a list of all amino acid residues whose $^1$H (H-N) chemical shifts were altered by at least 0.1 ppm at or near saturation binding of 2-aminoquinoline **2**. The absolute changes in $^1$H (H-N) chemical shift $|\Delta\delta|$ were then plotted against **[2]** (as illustrated in Figure A-1A below), and non-linear regression analysis performed using GraphPad Prism.[55] The calculated $K_d$ for each residue was then obtained from the Prism results table (Table A-2). The mean $\pm$ standard deviation of the obtained $K_d$ values was then calculated (Table A-1), to provide the $K_d$ value that has been quoted in the main text of this thesis (given in $\mu$M, following rounding to the nearest whole number). For ease of comparison between different ligands, $|\Delta\delta/\Delta\delta_{max}|$ terms were calculated for each residue (Table A-1). The mean $\pm$ standard deviation of these $|\Delta\delta/\Delta\delta_{max}|$ terms was then calculated (Table A-1) and this standardised data is plotted as in Figure A-1B below. Unless otherwise specified, this is the method by which equilibrium binding isotherms have been illustrated in the main text of this thesis.
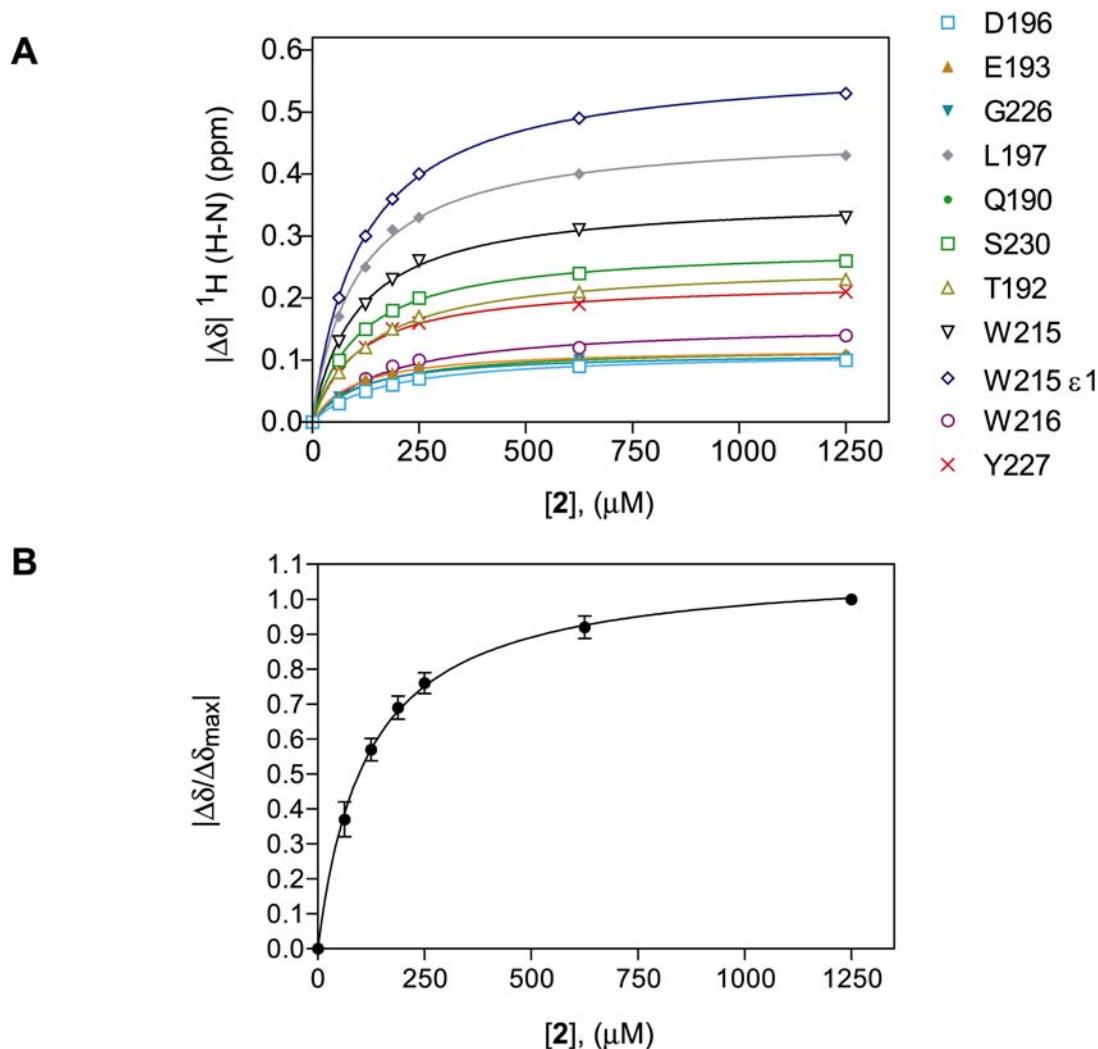


**Figure A-1:** Equilibrium binding of **2** to Tec SH3 domain as represented by change in $^1$H (H-N) chemical shift from [$^1$H,$^{15}$N]-HSQC NMR experiments. (A) Binding isotherms for amino-acid residues where $\Delta\delta$ = 0.1 ppm at close to saturation binding of **2**. (B) Binding isotherm represented by averaging the normalized chemical shift changes $|\Delta\delta/\Delta\delta_{max}|$ calculated for all residues where $|\Delta\delta_{max}|$ = 0.1 ppm. (See Table A-1).

**Table A-2:** Equilibrium binding of **2** to Tec SH3 domain using NMR. Summary of GraphPad Prism output following non-linear regression analysis of chemical shift changes for amino-acids residues involved in binding of **2** (Obtained from $\Delta\delta$ values presented in Table A-1 above.)

| Residue | D196 | E193 | G226 | L197 | Q190 | S230 |
|---|---|---|---|---|---|---|
| Equation 1 | | | | | | |
| Best-fit values | | | | | | |
| BMAX | 0.1135 | 0.1192 | 0.1126 | 0.4684 | 0.121 | 0.2838 |
| KD | 163.2 | 97.57 | 108.9 | 105.3 | 129.6 | 110.4 |
| Std. Error | | | | | | |
| BMAX | 0.001262 | 0.004195 | 0.002824 | 0.005674 | 0.0009455 | 0.002202 |
| KD | 5.461 | 12.54 | 9.539 | 4.515 | 3.309 | 2.975 |
| 95% Confidence Intervals | | | | | | |
| BMAX | 0.1103 to 0.1167 | 0.1084 to 0.1300 | 0.1053 to 0.1199 | 0.4538 to 0.4830 | 0.1185 to 0.1234 | 0.2781 to 0.2895 |
| KD | 149.1 to 177.2 | 65.33 to 129.8 | 84.35 to 133.4 | 93.65 to 116.9 | 121.1 to 138.1 | 102.7 to 118.0 |
| Goodness of Fit | | | | | | |
| Degrees of Freedom | 5 | 5 | 5 | 5 | 5 | 5 |
| R² | 0.9993 | 0.9906 | 0.9954 | 0.9989 | 0.9996 | 0.9996 |
| Absolute Sum of Squares | 5.03E-06 | 8.28E-05 | 3.48E-05 | 0.0001438 | 3.42E-06 | 2.09E-05 |
| Sy.x | 0.001003 | 0.00407 | 0.002638 | 0.005363 | 0.0008273 | 0.002046 |
| Data | | | | | | |
| Number of X values | 7 | 7 | 7 | 7 | 7 | 7 |
| Number of Y replicates | 1 | 1 | 1 | 1 | 1 | 1 |
| Total number of values | 7 | 7 | 7 | 7 | 7 | 7 |
| Number of missing values | 0 | 0 | 0 | 0 | 0 | 0 |

| Residue | T192 | W215 | W215e1 | W216 | Y227 |
|---|---|---|---|---|---|
| Equation 1 | | | | | |
| Best-fit values | | | | | |
| BMAX | 0.256 | 0.3627 | 0.5813 | 0.1588 | 0.2273 |
| KD | 135 | 108.8 | 116.4 | 168.4 | 108.1 |
| Std. Error | | | | | |
| BMAX | 0.002719 | 0.004506 | 0.002543 | 0.00932 | 0.003846 |
| KD | 4.616 | 4.723 | 1.733 | 29.45 | 6.409 |
| 95% Confidence Intervals | | | | | |
| BMAX | 0.2490 to 0.2630 | 0.3511 to 0.3743 | 0.5748 to 0.5878 | 0.1348 to 0.1827 | 0.2174 to 0.2371 |
| KD | 123.1 to 146.9 | 96.64 to 120.9 | 112.0 to 120.9 | 92.70 to 244.1 | 91.60 to 124.6 |
| Goodness of Fit | | | | | |
| Degrees of Freedom | 5 | 5 | 5 | 5 | 5 |
| R² | 0.9993 | 0.9989 | 0.9999 | 0.9819 | 0.9979 |
| Absolute Sum of Squares | 2.74E-05 | 8.86E-05 | 2.69E-05 | 0.0002664 | 6.49E-05 |
| Sy.x | 0.002341 | 0.004209 | 0.002317 | 0.007299 | 0.003601 |
| Data | | | | | |
| Number of X values | 7 | 7 | 7 | 7 | 7 |
| Number of Y replicates | 1 | 1 | 1 | 1 | 1 |
| Total number of values | 7 | 7 | 7 | 7 | 7 |
| Number of missing values | 0 | 0 | 0 | 0 | 0 |

**Table A-3:** FP peptide competition assay with **PRP-1** and **81** for Tec GST-SH3 protein.

| | | replicate 1 | | | replicate 2 | | | replicate 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PRP-1 alone mP** | | 74.56 | | | 71.38 | | | 77.73 | | |
| **[L] M** | **log[L]** | **mP** | **DmP** | **Proportion bound** | **mP** | **DmP** | **Proportion bound** | **mP** | **DmP** | **Proportion bound** |
| 0.00E+00 | | 132.75 | 58.19 | 1.00 | 130.87 | 59.49 | 1.00 | 129.00 | 51.26 | 1.00 |
| 7.50E-07 | -6.12 | 133.22 | 58.66 | 1.01 | 127.78 | 56.40 | 0.95 | 128.11 | 50.37 | 0.98 |
| 1.00E-06 | -6.00 | 129.83 | 55.27 | 0.95 | 132.88 | 61.50 | 1.03 | 126.78 | 49.04 | 0.96 |
| 3.16E-06 | -5.50 | 126.11 | 51.56 | 0.89 | 132.92 | 61.53 | 1.03 | 135.18 | 57.44 | 1.12 |
| 1.00E-05 | -5.00 | 121.86 | 47.30 | 0.81 | 125.67 | 54.28 | 0.91 | 121.61 | 43.88 | 0.86 |
| 3.16E-05 | -4.50 | 123.19 | 48.63 | 0.84 | 117.92 | 46.54 | 0.78 | 110.36 | 32.63 | 0.64 |
| 1.00E-04 | -4.00 | 87.33 | 12.77 | 0.22 | 84.76 | 13.38 | 0.22 | 87.36 | 9.63 | 0.19 |
| 3.50E-04 | -3.46 | 78.81 | 4.25 | 0.07 | 83.53 | 12.14 | 0.20 | 81.72 | 3.99 | 0.08 |
| 7.00E-04 | -3.15 | 77.74 | 3.18 | 0.05 | 78.47 | 7.09 | 0.12 | 77.17 | -0.57 | -0.01 |
| 1.00E-03 | -3.00 | 87.92 | 13.36 | 0.23 | 80.05 | 8.67 | 0.15 | 80.23 | 2.49 | 0.05 |
| 1.35E-03 | -2.87 | 79.77 | 5.21 | 0.09 | 72.19 | 0.80 | 0.01 | 72.22 | -5.51 | -0.11 |

| **Summary** | | **Proportion bound** | | | **Average of** | **St Dev of** | | |
|---|---|---|---|---|---|---|---|---|
| | **log[L]** | **replicate 1** | **replicate 2** | **replicate 3** | **Replicates** | **Replicates** | | |
| | -6.12 | 1.01 | 0.95 | 0.98 | 0.98 | 0.03 | | |
| | -6.00 | 0.95 | 1.03 | 0.96 | 0.98 | 0.05 | | |
| | -5.50 | 0.89 | 1.03 | 1.12 | 1.01 | 0.12 | | |
| | -5.00 | 0.81 | 0.91 | 0.86 | 0.86 | 0.05 | | |
| | -4.50 | 0.84 | 0.78 | 0.64 | 0.75 | 0.10 | | |
| | -4.00 | 0.22 | 0.23 | 0.19 | 0.21 | 0.02 | | |
| | -3.46 | 0.07 | 0.20 | 0.08 | 0.12 | 0.07 | | **EC$_{50}$** |
| | -3.15 | 0.06 | 0.12 | -0.01 | 0.05 | 0.07 | | |
| | -3.00 | 0.23 | 0.15 | 0.05 | 0.14 | 0.09 | **Average** | **(mM)** 51.34 |
| | -2.87 | 0.09 | 0.01 | -0.11 | 0.00 | 0.10 | **StDev** | **(mM)** 4.17 |
| **CALCULATED EC$_{50}$ (M)** | | 5.35E-05 | 5.40E-05 | 4.65E-05 | | | | |

Table A-3 above contains a list of the millipolarisation (mP) values for three replicate experiments involving the competition between the binding of **PRP-1** and ligand **81** for the GST-SH3 protein, at different concentrations of **81** (refer to Section 7.7.2 for definition of mP, and other equations relevant to this discussion). For each replicate, $\Delta$mP values were calculated, followed by proportion bound terms (Table A-3). The proportion bound terms were then plotted against log[**81**] for each replicate (Figure A-2A), and non-linear regression analysis using GraphPad Prism used to determine $EC_{50}$ values (Table A-4). The mean $\pm$ standard deviation of each $EC_{50}$ value provided the $EC_{50}$ value as quoted in the main text of this thesis (given in $\mu$M, following rounding to the nearest whole number). For ease of comparison between different ligands, the mean $\pm$ standard deviation of the proportion bound terms (produced for the individual replicates) was calculated (Table A-3), and this data plotted against log[**81**] as illustrated in Figure A-2B. This is the method by which the competition binding isotherms have been illustrated in the main text of this thesis.
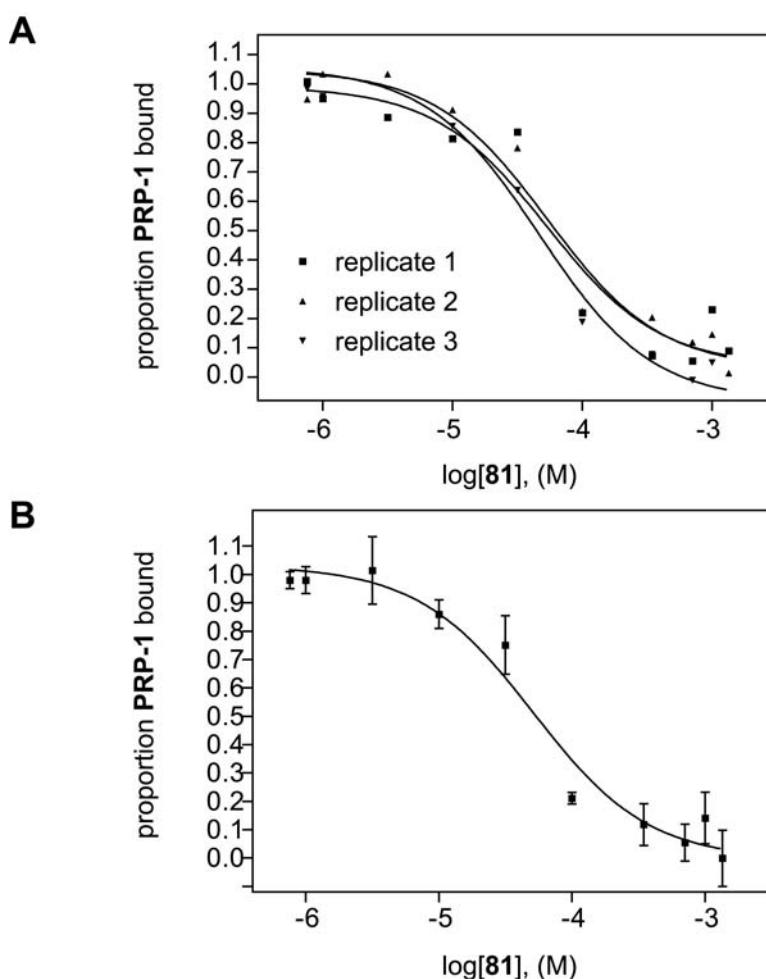


**Figure A-2:** FP peptide competition assay for **PRP-1** and **81** for binding to Tec GST-SH3 protein. (A) Competition isotherm for individual replicate data sets produced from data presented in Table A-3 above. (B) Competition isotherm for average of replicates, produced from data presented in Table A-3 above.

**Table A-4:** Summary of FP peptide competition assay with **PRP-1** and **81** for binding to Tec SH3 domain. [Output obtained from non-linear regression analysis of data in Table A-3 and curves in Figure A-2 (A) above.]

| | replicate 1 | replicate 2 | replicate 3 |
|---|---|---|---|
| Equation 1 | | | |
| Best-fit values | | | |
|     BOTTOM | 0.03615 | 0.02892 | -0.08215 |
|     TOP | 0.991 | 1.048 | 1.057 |
|     LOGEC50 | -4.271 | -4.268 | -4.332 |
|     EC50 | 5.36E-05 | 5.40E-05 | 4.65E-05 |
| Std. Error | | | |
|     BOTTOM | 0.0798 | 0.06049 | 0.05375 |
|     TOP | 0.07344 | 0.05548 | 0.0526 |
|     LOGEC50 | 0.2238 | 0.1587 | 0.1301 |
| 95% Confidence Intervals | | | |
|     BOTTOM | -0.1526 to 0.2249 | -0.1141 to 0.1720 | -0.2093 to 0.04497 |
|     TOP | 0.8174 to 1.165 | 0.9169 to 1.179 | 0.9323 to 1.181 |
|     LOGEC50 | -4.800 to -3.742 | -4.643 to -3.893 | -4.640 to -4.025 |
|     EC50 | 1.5830e-005 to 0.0001811 | 2.2740e-005 to 0.0001280 | 2.2910e-005 to 9.4500e-005 |
| Goodness of Fit | | | |
|     Degrees of Freedom | 7 | 7 | 7 |
|     $R^2$ | 0.9346 | 0.966 | 0.9766 |
|     Absolute Sum of Squares | 0.09929 | 0.0568 | 0.04865 |
|     Sy.x | 0.1191 | 0.09008 | 0.08337 |
| Data | | | |
|     Number of X values | 10 | 10 | 10 |
|     Number of Y replicates | 1 | 1 | 1 |
|     Total number of values | 10 | 10 | 10 |
|     Number of missing values | 0 | 0 | 0 |

# Appendix 4

Published article:

Inglis et al., (2004) 'Identification and specificity studies of small-molecule ligands for SH3 protein domains'. *J. Med. Chem.*, 47 (22), pp. 5405-5417