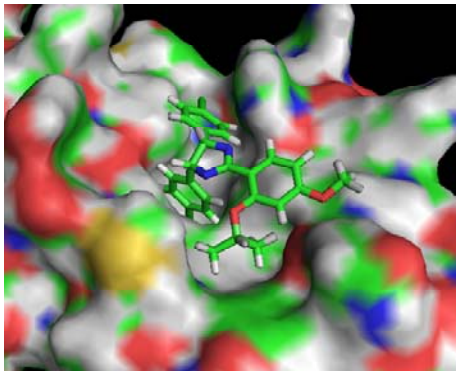
Inhibition of the p53-MDM2 Interaction

Introduction

The interaction between MDM2 and p53 is of great interest to cancer biologists as p53 has a vast number of cellular targets. P53 can both be activated and activate a large number of pathways, leading to a halt in the cell cycle or even apoptosis. These effects are mediated either directly by p53, for example direct interaction with the pro-apoptotic factors Bak and Bax or transcriptionally, for example leading to the transcription of p21 that inhibits cyclin dependent kinases and hence prevents cell cycle progression.

MDM2 is an oncogene that binds to p53 and inactivates it, by direct blocking of activity; nuclear export; or even ubiquitin ligation via its E3 ligase activity to target p53 for degradation. If one blocks this interaction then p53 activity is increased as it is neither inactivated nor destroyed and hence this interaction is of therapeutic importance in halting the growth or even selectively destroying tumours.

A vast number of inhibitors have been produced using various screening methods both *in vitro* and *in silico* in order to produce an inhibitor with sufficient affinity. These inhibitors are of great importance as very few small molecule inhibitors of protein-protein interactions have been produced: it was possible to target this interaction due to the relatively small interaction surface area. One possible inhibitor is shown resembles on of the nutlin family of inhibitors and by making three hydrophobic contacts mimics the MDM2-binding region of p53. Phe19, Trp23 and Leu26 of p53 insert into the hydrophobic grooves shown and hence many inhibitors involve groups similar to the amino acids involved in the protein-protein interaction.



In this project I was to characterize the binding affinities of the inhibitors to MDM2 in order to compare to the NMR titrations and IC50 values calculated via an ELISA competition assay. I was to use a variety of techniques such as ITC, SPR and fluorescence; however it quickly emerged that the fluorescence technique was of most use, especially in this case as the sequence of MDM2 (appendix 1.) showed that there were no tryptophan residues and hence it was possible to, at certain excitation wavelengths make MDM2 essentially invisible.

Experimental Procedure

Using the Perkin Elmer Luminescence Spectrometer LS50B measure between excitation wavelengths 230-300nm, measuring in each case the emission between 300 and 400nm[1] of each inhibitor. This is compared for each

[1] This was achieved using a 10nm min^{-1} scan with 5nm increments such that there were 15 scans of approximately ten minutes each.

inhibitor with 10μM MDM2 alone in order to find a wavelength at which the emission by MDM2 is minimal and the inhibitor emission is sufficient to allow an adequate signal: noise ratio. A single wavelength of excitation is chosen for each inhibitor and the effect on the fluorescence profile in observed. This could, most commonly be a change in the emission wavelength max or in a few rare cases the change in linewidth resulting from peak narrowing or broadening: all of which can be determined using graphical software, in this case the Lorentz fit on Origin 7.

By using the calculated IC50 values a suitable range of MDM2 concentrations can be selected, approximately from tenfold below (in addition to the zero point) to tenfold above, with each MDM2 concentration being double the previous. The concentration of the inhibitor was also selected from this IC50 such that the concentration allows any effect to be due to binding rather than saturation and for this reason the concentrations selected were not more than 10-fold greater than the IC50. This leads to a small dilution effect; however since I was not observing the intensity this small effect could be ignored, as it would not affect the wavelength max or linewidth significantly. The number of scans used varied (I chose to scan at 100nm min^{-1}) depending on the signal strength, for example when an inhibitor concentration was 10μM then there were in general fewer scans needed in order to achieve the same quality data: this is summarized below.

By then finding the difference between the maximum emission wavelength/linewidth and that at 0μM MDM2 it was possible to plot MDM2 concentration (x-axis) against either the change in emission maximum peak or change in linewidth and fit a binding curve to these data, thus calculating a value for Kd.

Note that there were two alternate nomenclature systems used therefore in the tables below I have included both; however I will use the 8* system.

Inhibitor		Inhibitor concentration	MDM2 range	Excitation wavelength	Scans (nm min-1)
8222	Ib	10μM	0.40-12.03μM	295	10 x 100
8230	Ia''				
8231	Ia	10μM	0.40-16.93μM	245	40 x 100
8247	IIIc	10μM	0.79-16.93μM	235	40 x 100
8248	IIIc'	10μM	0.05-3.01μM	235	40 x 100
8251	nutlin-3				
8253	Ic	10μM			
8261	Iic	1μM			
8262	IIIa	1μM	0.10-3.01μM	255	40 x 100
8271	IIIb	10μM	0.05-3.01μM	235	40 x 100
8348	Iib	1μM	0.05-	235	40 x 100

			1.55µM		
8349	IIa	1µM	0.05-0.79µM	235	40 x 100
8354 mix					
8354A					
8354B					
p53peptide		1µM	0.11-13.05µM	295	10 x 100

Results

Inhibitor		Kd	Bmax	IC50 (µM)	NMR titration L82/R29 (µM)	
8222	Ib	**4.925±1.723**	**4.35±0.69**	16.4±1.6	1.53	1.53
8230	Ia''			41.6±7.8	13.1	14
8231	Ia	**7.894±1.7823**	**27.68±2.93**	5.3±0.9	14	24.2
8247	IIIc	**2.086±0.397**	**21.53±1.11nm**	5±1	48.8	48.8
8248	IIIc'	**0.732±0.158**	**63.87±5.27nm**	4±1		
8251	nutlin-3			0.04±0.003		
8253	Ic			3±0.7	49.9	
8261	Iic					
8262	IIIa	**0.162±0.0045**	**19.98±1.01**	0.96±0.03	56.7	54.5
8271	IIIb	**0.713±0.105**	**3.46±0.19**	3.4±0.7	278	102
8348	Iib	**0.641±0.278**	**5.81±1.14**	0.532±0.074	14	10
8349	Iia	**0.399±0.070**	**96.41±7.76**	0.357±0.044	1.53	1.53
8354 mix						
8354A						
8354B						
p53peptide		**1.084±0.049**	**17.65±0.23**	0.06-0.7		

The results obtained do not agree closely with the NMR titration but fit more closely with the IC50 values; however I will discuss each in detail.

P53 peptide- This was my first experiment in order to test my experimental setup; however it showed that my fluorescence results are similar to the expected IC50 values but the Kd is slightly higher than one might expect.

8222 - The calculated Kd lies between the IC50 and the NMR titration values and could illustrate the differences between experimental procedures.

8230 - Unfortunately the attempts with 8230 were unsuccessful due to the relatively low signal despite the use of 10μM solution coupled with the fact that high levels of MDM2 were required and hence the contribution could no longer be ignored.

8231- This inhibitor gave results where the fluorescence and IC50 value were within their confidence limits and hence shows good agreement for an affinity. The NMR titration data gave approximately tenfold higher values for the Kd.

8247 – The IC50 and fluorescence results were in the same order of magnitude and again the Kd from the NMR titration was over tenfold greater than both of these.

8248 – The calculated Kd was approximately fivefold lower than the IC50 given; however this may have been due to the inhibitor concentration being greater than tenfold the Kd, hence producing a less reliable measure that could have involved saturation rather than binding. A short repeat experiment with 5μM 8248 using few points seemed to agree with the 0.7μM Kd; however a full repeat experiment is suggested in order to confirm this.

8251 – This inhibitor is also known as nutlin-3 and from previous experiments is likely to have a Kd of around 40nM. One of our experiments using 1μM 8251 (that in itself could lead to unreliable results) showed a likely Kd of 36±4nM that fits with already known data. Unfortunately there was a contaminant that seemed not to affect affinity; however on an attempt to repeat this experiment without the contaminant 0.1μM 8251 was chosen yet this gave unsatisfactory results and owing to the lack of NMR data and time constraints this was not pursued further.

8253 – This was attempted a few different times; however the peaks at saturation were affected by MDM2 and hence no saturation was seen; however from the initial points it is likely that the Kd lies between 3 and 6 μM that would agree with the IC50 but be tenfold lower than the NMR data.

8261 – There were also issues here for the same reasons and so as there were no NMR or IC50 values and my approximate Kd was over 6μM there was no pressing reason to focus strongly on this inhibitor.

8262 – A much tighter binding inhibitor according to the fluorescence and IC50 values but once again there is a huge difference between these and the NMR titration that had a much higher Kd of over 50-fold the IC50 and 250-fold the fluorescence data.

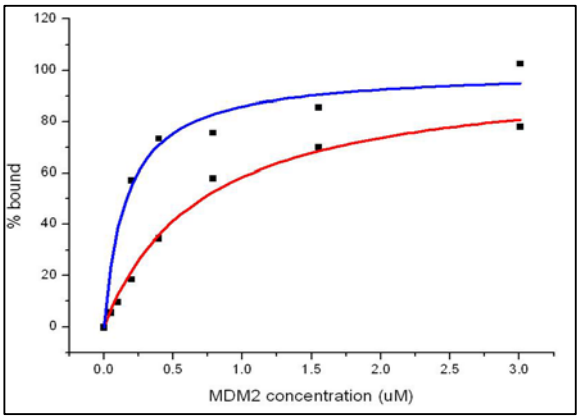
8271 – The data obtained for this inhibitor was fivefold below the IC50 value and over a hundredfold lower than the NMR titration data. In this case as with 8248 10μM was initially used as an inhibitor concentration so a saturation effect may have been caused. This was tested in a similar manner to 8248, giving a value of 1.66±0.42μM that is closer to the IC50 value but is not far beyond the initial 0.7μM Kd.

8349 – The calculated Kd was extremely close to the IC50 value and only fivefold less than the NMR titration result, making this the inhibitor that the three techniques agree most closely on.

8354 – This is an interesting inhibitor as it seemed to have a very low Kd; however this proved too low to measure at 0.1μM inhibitor concentration due to the high signal to noise ratio. This is a good inhibitor to pursue with other techniques more suited for very tight binding inhibitors.
The two enantiomers of 8354 (A and B) were also to be measured; however due to the difficulties with the racemic mixture neither enantiomer was measured in isolation.

General trends

The vast majority of measured Kd's were within the same order of magnitude as the IC50 values and so the fluorescence technique has proven to be a valuable alternative technique to use in order to calculate affinities. The NMR titrations were generally at least fifty fold larger than the fluorescence results; however this may be the result of the difficulty of NMR to measure very tight binding inhibitors. Most of the techniques agreed which inhibitors bound the tightest, for example the substitution of –Cl in 8271 (red) being replaced with $-N0_2$ in 8262 (blue) that increased the affinity 2 or 3 fold. This is an example of structure aided design as this larger group fits more snuggly in the hydrophobic pocket of MDM2 thus making more Van der Waal's contacts.



Appendix

1. **MDM2 sequence**

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MCNTNMSVPT DGAVTTSQIP ASEQETLVRP KPLLLKLLKS VGAQKDTYTM
KEVLFYLGQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKEHRKIY
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HLVSRPSTSS RRRAISETEE NSDELSGERQ
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