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Mechanistic Studies on Hsp90 Inhibition by Ansamycin Derivatives

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Heat shock protein 90 (Hsp90) is a molecular chaperone that is required for the maturation and activation of a number of client proteins, many of which are involved in cancer development. The ansamycin family of natural products and their derivatives, such as geldanamycin (GA), are well-known inhibitors of the essential ATPase activity of Hsp90. Despite structural studies on the complexes of ansamycin derivatives with the ATPase domain of Hsp90, certain aspects of their inhibitory mechanism remain unresolved. For example, it is known that GA in solution exists in an extended conformation with a *trans* amide bond; however, it binds to Hsp90 in a significantly more compact conformation with a *cis* amide bond. GA and its derivatives have been shown to bind to Hsp90 with low micromolar affinity *in vitro*, in contrast to the low nanomolar anti-proliferative activity that these drugs exhibit *in vivo*. In addition, they show selectivity towards tumour cells. We have studied both the equilibrium binding, and the association and dissociation kinetics of GA derivative, 17-DMAG, and the fluorescently labelled analogue BDGA to both wild-type and mutant Hsp90. The mutants were made in order to test the hypothesis that conserved residues near the ATP-binding site may catalyse the *trans-cis* isomerisation of GA. Our results show that Hsp90 does not catalyse the *trans-cis* isomerisation of GA, and suggests that there is no isomerisation step before binding to Hsp90. Experiments with BDGA measured over a wide range of conditions, in the absence and in the presence of reducing agents, confirm recent studies that have suggested that the reduced dihydroquinone form of the drug binds to Hsp90 considerably more tightly than the non-reduced quinone species.

Keywords: ITC; fluorescence anisotropy; *trans-cis* isomerisation; association kinetics; dissociation kinetics

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The cytosolic molecular chaperone heat-shock protein 90 (Hsp90) is involved in the maturation and activation of a number of proteins, collectively known as client proteins. Many proteins that depend upon Hsp90 to attain their correctly folded, active conformation are involved in signal-transduction pathways, cell-cycle regulation and apoptosis pathways, which are commonly deregulated in cancers.[1–3] Consequently, and despite its importance for cellular viability, Hsp90 has been shown to be an attractive target for cancer therapeutics.[4,5]

Geldanamycin (GA), a naturally occurring benzoquinone ansamycin, is one of a number of Hsp90 inhibitors that has shown promise as a chemotherapeutic agent. GA binds to and inhibits Hsp90 by preventing binding and subsequent hydrolysis of ATP, which is essential for protein function.[6] GA has been shown to reduce cellular levels of client proteins that are essential for cancer cell survival, such

Abbreviations used: GA, geldanamycin; BDGA, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-geldanamycin; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; DMAG, 17-dimethyl-aminoethyl-17-demethoxygeldanamycin; ITC, isothermal calorimetry; TCEP, tris-(2-carboxyethyl) phosphine.

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as mutated p53, Akt, Bcr-Abl, and ErbB2.[7] Two GA derivatives, 17-allylamino,17-demethoxygeldanamycin (17-AAG) and the water-soluble 17-dimethylaminoethyl,17-demethoxygeldanamycin (DMAG), are currently in phase II and phase I clinical trials, respectively.[4,8,9]

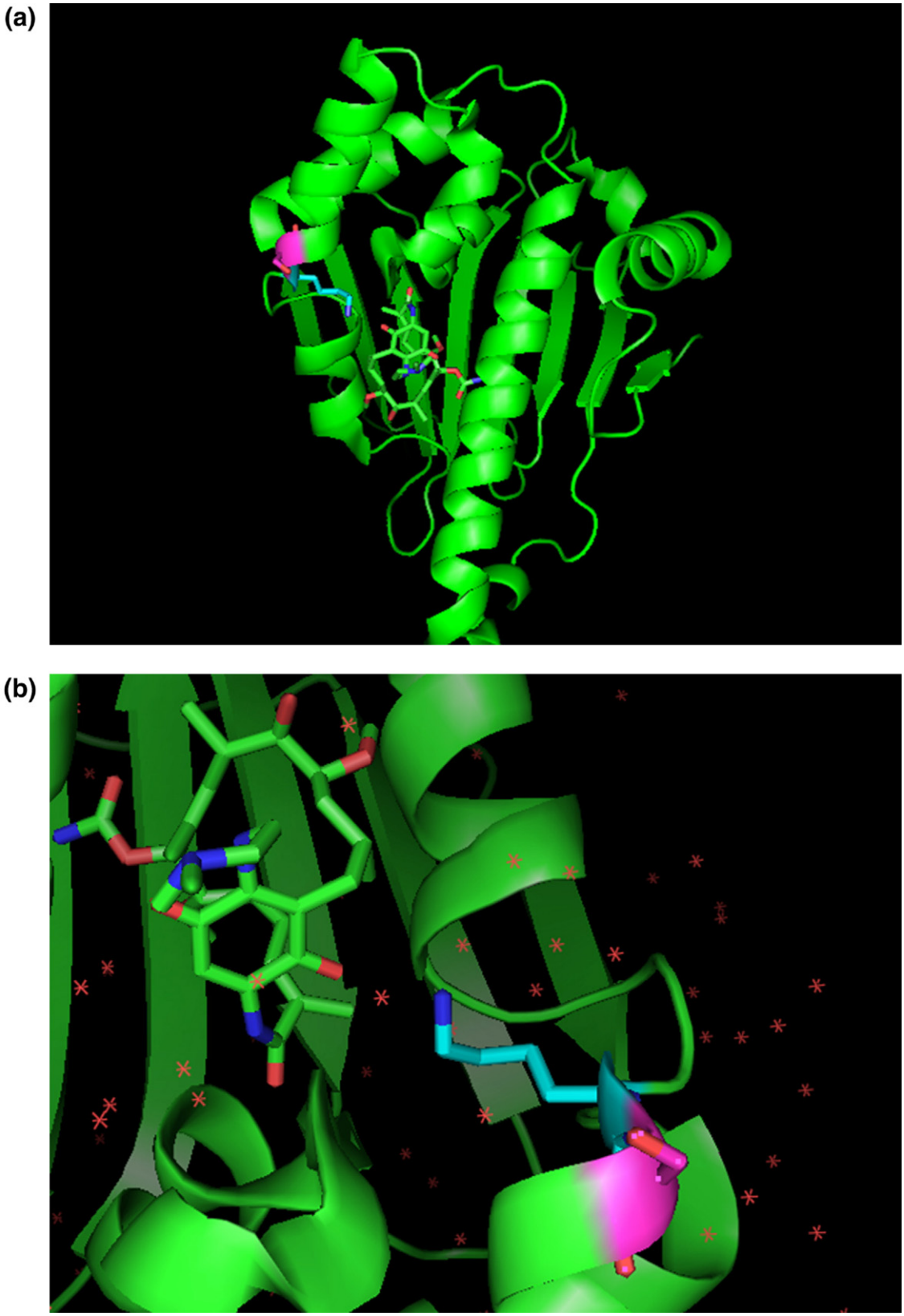


Figure 1. Crystal structure showing DMAG in complex with (a) Hsp90, residues Lys106 (cyan) and Ser107 (pink) are highlighted; (b) water molecules involved in the binding site are shown (red stars).

Geldanamycin and its derivatives have been shown to bind to Hsp90 *in vitro* with low micromolar affinity.[10] However, this is in stark contrast to the low nanomolar anti-proliferative activity that the drug exhibits *in vivo*.[11] The underlying cause of the difference in affinity between purified Hsp90 *in vitro* and Hsp90 expressed in cancerous cells/tissue *in vivo*, and the apparent selectivity of the drug towards tumour cells, has been the subject of much debate.[11,12] In recent years, a number of theories on the origin of these differences have been proposed. Kamal and co-workers provided biochemical evidence that Hsp90 in cancer cells exists primarily in a multi-chaperone complex that has a higher affinity for GA than Hsp90 in normal cells, where the majority of the protein is not present in a high molecular mass complex, or purified Hsp90.[13] However, this observation has been challenged recently by several groups.[14–16] It has been suggested that Hsp90 undergoes a slow conformational change upon binding of GA, leading to a tight interaction. The slow, tight-binding of GA has been proposed as a possible explanation for the accumulation of GA in tumour cells due to the law of mass action, thereby causing an increase in the apparent affinity of tumour-specific Hsp90.[14] In a different study, it has been proposed that reduction of the quinone moiety in GA may have an important role in enhanced binding and selectivity.[15] Unlike the structurally distinct Hsp90 inhibitor radicicol, which does not need to change conformation on binding to Hsp90, the binding of GA causes the extended conformation with a *trans* amide bond to change to a kinked, C-shaped conformation where the amide group in the ansa ring has a *cis* configuration.[10] A detailed quantum chemical analysis of this isomerisation step has recently led to a model where Hsp90 itself acts as a *trans-cis* isomerase of GA, catalyzing the conversion of the *trans* unbound form to the more compact *cis* Hsp90-bound form.[16] This study identified two conserved residues near the ATP-binding pocket (equivalent to residues Lys106 and Ser107 in human Hsp90β), which were proposed to be involved in the isomerase activity. These residues were mutated in chicken Hsp90 and, on the basis of the observation that the K112A mutant showed reduced affinity for GA, and that the S113A mutation effectively blocked binding of GA to Hsp90, a mechanism was proposed in which these residues catalyse the conversion of *trans* to *cis* GA.[16] The requirement of isomerisation of GA before tight binding has been postulated as a possible reason behind the enhanced binding affinity of GA for Hsp90 *in vivo* compared to its purified form *in vitro*.[16]

In this study, we have investigated the thermodynamics and kinetics of binding of GA derivatives to wild-type and mutant human Hsp90 under a wide range of experimental conditions. We have tested a number of the hypotheses discussed above, including (i) the tight binding of GA *in vivo* is due to a slow conformational change that is not detected in the currently widely used assays of ligand binding where protein–ligand samples are incubated for only fairly short periods (several minutes); (ii) that residues Lys106 and Ser107 have an important role in GA isomerisation; and (iii) that reduction of the quinone ring results in tighter binding of the drug to Hsp90. In contrast to previous studies, we find no evidence for a slow, tight-binding event, and both equilibrium and kinetic studies are consistent with a simple, single-step process. In addition, and again in contrast to previous studies, we find no evidence to suggest that Hsp90 acts as a catalyst of the *trans-cis* isomerisation of GA. We also confirm the results of earlier studies and show that the reduction of the quinone moiety of GA to its dihydroquinone, which can be brought about by small quantities of reducing agents typically present in assay buffers, leads to increased affinity.

Lysine 106 and serine 107 in human Hsp90 (Figure 1) were mutated to alanine in order to investigate the effects that these residues have on GA binding to Hsp90. Isothermal titration calorimetry (ITC) was used to determine the dissociation constants (K_d) and stoichiometry of ligands binding to wild-type and mutant Hsp90 (Figure 2 and Table 1). Consistent with previously measured values for GA derivatives,[9,10] wild-type human Hsp90 bound DMAG with a K_d of 0.35(±0.04) μM (Figure 2(a)). The K106A mutant bound DMAG with a K_d of 1.40(±0.08) μM (Figure 2(b)), a four-fold reduction in affinity compared to the wild type, whilst the S107A mutant bound DMAG with a K_d of 0.48(±0.07) μM (Figure 2(c)), which is comparable to that of wild-type. In each case, the stoichiometry of binding was close to a 1:1 ratio of ligand to Hsp90 monomer, as expected. From the X-ray crystal structures of Hsp90 in complex with GA,[10] Lys106 is expected to interact with DMAG *via* a water molecule (Figure 1(b)). Thus, the observed reduction in binding affinity for DMAG upon mutation of this residue is consistent with structural studies and agrees with previous observations.[16] Previous work, however, has shown that mutation of Ser113 in chicken Hsp90 abolishes GA binding. In contrast to this, and again consistent with structural work, we observe no dramatic reduction in binding affinity for the S107A mutant, which has a K_d similar to that of the wild type.

The binding affinity of the fluorescently labeled GA analogue 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene-geldanamycin (BDGA) to wild-type and mutant Hsp90 was measured using fluorescence anisotropy.[17] In this case, wild-type Hsp90 bound BDGA with a K_d of 29(±3) nM, following incubation for 24 h (Figure 2(d)). S107A bound BDGA with an affinity of 63(±9) nM, while K106A bound BDGA with a K_d of 117(±13) nM. Although the observed binding of all forms of Hsp90 to BDGA was tighter than that of DMAG as measured by ITC (the origin of the differences in the binding constants determined by ITC and fluorescence anisotropy measurements is addressed in detail later), the relative binding constants and effect of the mutations was similar to the ITC experiments. K106A showed a

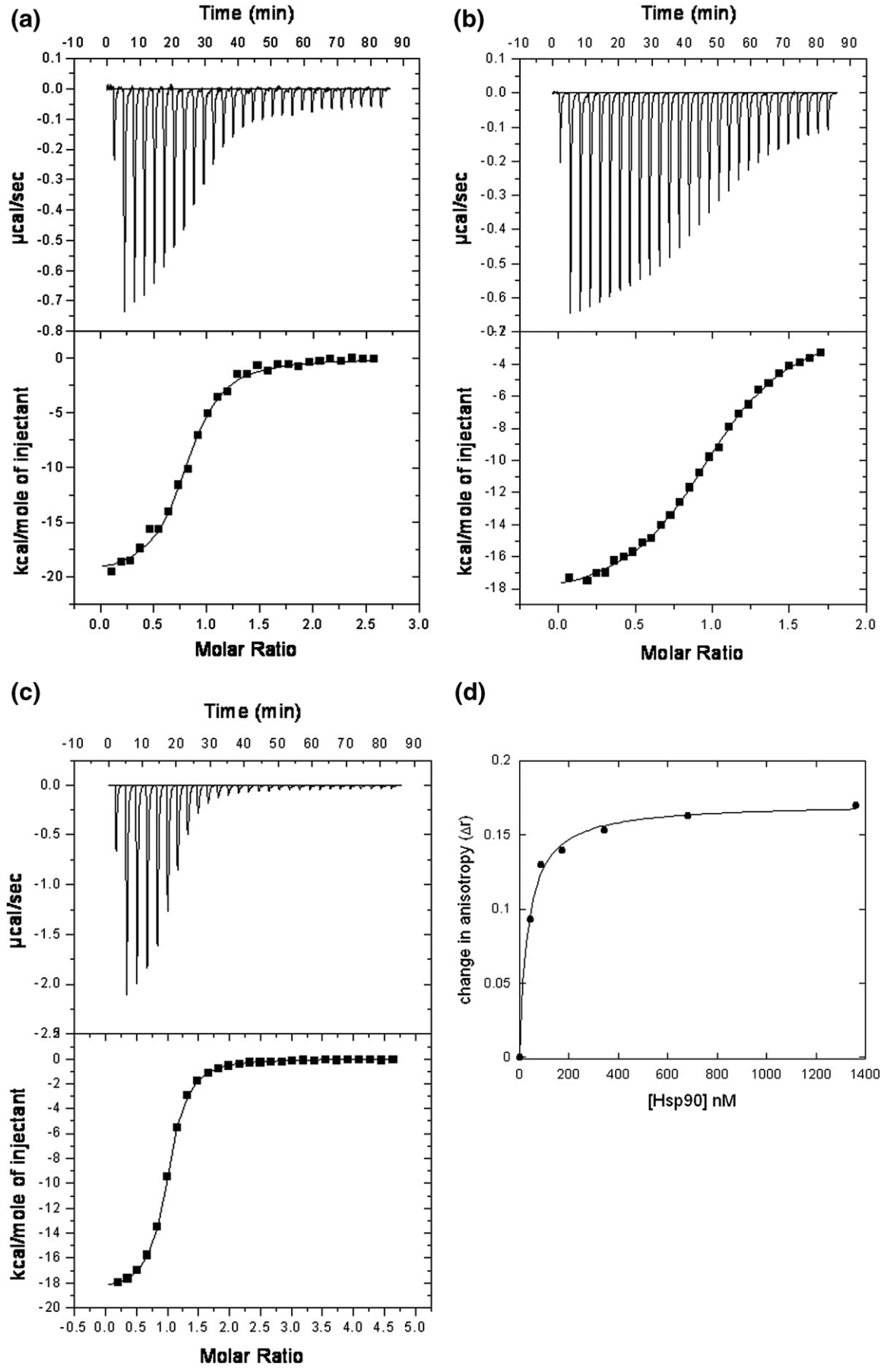


Figure 2 (*legend on next page*)

larger reduction in binding affinity relative to wild-type compared to S107A. The equilibrium binding results obtained in this study are in contrast to results seen in previous studies; however, they are consistent with the crystal structures of Hsp90-GA complexes.[10]

Serine 107 has been suggested to be of importance in the catalysis of *trans* to *cis* GA, by acting as a docking site for the binding of *trans* GA, and catalysing the conversion to the *cis* isoform.[16] It is possible, therefore, that mutation of this residue may differentially affect association and dissociation rates. To investigate this, we determined the association (k_{on}) and dissociation (k_{off}) rate constants of DMAG binding to wild-type and mutant Hsp90 using stopped-flow fluorescence (Figure 3). Following the rapid mixing of Hsp90 and DMAG, the intrinsic fluorescence of Hsp90 decreases exponentially and data were fit to a single-exponential function, from which apparent association rate constants were obtained. The observed rate constants at different concentrations of DMAG were fit to a simple linear equation: $k_{obs}=k_{on}[L]+k_{off}$, where [L] is the concentration of DMAG (Figure 3). The intercept of the plot gives a value for k_{off}, and the gradient gives k_{on}. The k_{on} for DMAG binding to wild-type Hsp90 was $8\times10^4(\pm1\times10^4)$ M^{-1} s^{-1}, whilst the k_{off} was 0.046(±0.001) s^{-1}. A dissociation constant, K_d, can be determined from the ratio of k_{off} and k_{on} ($K_d=k_{off}/k_{on}$) and for wild-type Hsp90 this was 0.57(±0.07) μM. The association and dissociation rate constants for S107A and K106A were $6.7\times10^4(\pm1\times10^4)$ M^{-1} s^{-1} and 0.050(±0.001 s^{-1}), and $2.1\times10^5(\pm0.9\times10^5)$ M^{-1} s^{-1} and 0.294(±0.012) s^{-1}, respectively, and the corresponding values of K_d for K106A and S107A were 1.44(±0.63) μM and 0.75(±0.15) μM, respectively. In all cases, the K_d values calculated from the kinetic rate constants agreed well with the values obtained from the ITC experiments, suggesting that there is no second step in the binding of DMAG to Hsp90 under these conditions. In addition, whilst wild-type Hsp90 and the S107A mutant have comparable values for k_{on} and k_{off}, the K106A mutant has significantly different rate constants. The increase in k_{off} observed for K106A is to be expected, as the mutation weakens the interaction between GA derivatives and Hsp90 (Figure 1(b)), as shown by ITC measurements. However, the significant increase in k_{on} observed for K106A was somewhat unexpected. One possible explanation for this may be the presence of a tertiary amine group in DMAG, which would have a pK_b of approximately 10.5, and therefore be positively charged at neutral pH. The positively charged Lys106 is likely, therefore, to make unfavourable electrostatic interactions with this particular ligand. Neutralisation of the positive charge on Lys106 on mutation thus favours association and leads to the increased k_{on} observed.

In these experiments, k_{off} is determined from the *y*-intercept of the plot of apparent association rate constant *versus* ligand concentration. However, determination of k_{off} using this method is subject to large errors, as it involves extrapolation to values very close to zero. Therefore, values of k_{off} were determined in a separate experiment by observing the change in intrinsic fluorescence upon dissociation of DMAG from Hsp90 (Figure 3 and Table 1). Once again, data were fit to a single-exponential equation to obtain a value for k_{obs}, the observed dissociation rate constant. Under the conditions used, in which complete dissociation of DMAG does not occur, the observed rate constant is not equal to k_{off}; however, the relative values of k_{obs} measured at time zero and at 24 h after incubation of the Hsp90–DMAG complex (Table 1) are a good indicator of the effect of longer incubation times in the presence of different reducing agents on the dissociation rates. The data agreed well with results obtained from other stopped-flow experiments, with K106A exhibiting a significantly increased k_{off} compared to wild-type, while the k_{off} of the S107A mutant was close to the wild-type value (Table 1).

It has been suggested that ansamycin binding to Hsp90 occurs in a two-step process, in which Hsp90 and GA derivatives initially form a weak encounter complex, with an apparent K_d in the low micromolar range.[14] The ligand-bound Hsp90 then undergoes a slow conformational change, leading to a state that has an increased affinity for GA.[14] To investigate this, we monitored the change in fluorescence anisotropy upon binding of BDGA to Hsp90 over time in our standard assay buffer (Figure 4, Table 1). Data were fit to equation (1),

Figure 2. Isothermal titration calorimetry of DMAG binding to (a) wild-type, (b) K106A and (c) S107A Hsp90. DMAG was injected into a cell containing full-length Hsp90 at 37 °C. Wild-type Hsp90 and both mutants bound DMAG with stoichiometries of approximately 1:1 and with dissociation constants of 350 nM, 1402 nM and 477 nM for wild-type, K106A and S107A, respectively. (d) Typical fluorescence anisotropy data for the binding of BDGA to wild-type Hsp90. Wild-type and mutant (generated by site-directed mutagenesis, Quick-Change, Stratagene), human Hsp90β were expressed and purified as described.[20] Protein was greater than 95% pure. Protein concentrations were determined spectrophotometrically using an extinction coefficient ε_{280} of 53,360 M^{-1} cm^{-1} and are quoted as monomers. Isothermal titration calorimetry was performed using a MicroCal VP-ITC instrument (Microcal Inc., Northampton, MA USA). Samples (300 μl) of 110 μM DMAG, 105 μM DMAG, and 362 μM DMAG were injected into a 1.4 ml cell containing 9 μM wild-type Hsp90, 12.5 μM K106A and 16 μM S107A, in 50 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$ 20 mM KCl, 0.25 mM β-mercaptoethanol (assay buffer) at 37 °C. Similarly, a sample of 300 μM ADP was injected into 22 μM K106A and S107A. Parallel experiments were carried out by injecting DMAG or nucleotide into assay buffer without Hsp90 to correct for the heat of dilution in the subsequent data analysis using the Origin software package (MicroCal Inc.). The concentrations of protein and nucleotide were determined spectrophotometrically post injection. β-Mercaptoethanol does not reduce GA or its derivatives under the conditions used (data not shown).

Table 1. Summary of the thermodynamic and kinetic parameters for ligand binding to Hsp90

	Ligand	Reducing agent	K_d (μM)	K_d (nM) 0.12 h	K_d (nM) 1 h	K_d (nM) 2 h	K_d (nM) 4 h	K_d (nM) 8 h	K_d (nM) 24 h	k_{on} ($\mu M^{-1} s^{-1}$)	k_{off}[a] (s^{-1})	K_d (k_{off}/k_{on}) (μM)
ITC/anisotropy experiments												
Wild-type	DMAG[b]		0.35±0.04							0.08±0.01	0.046±0.001	0.57±0.07
	DMAG (reduced)[b]		0.0022±0.0014									
	AMPPNP[b]		141									
	ADP[b]		15									
	BDGA[c]		0.029±0.003									
K106A	DMAG[b]		1.40±0.08							0.21±0.09	0.294±0.012	1.4±0.6
	AMPPNP[b]		76±11									
	ADP[b]		15.1±2.4									
	BDGA[c]		0.17±0.03									
S107A	DMAG[b]		0.48±0.07							0.07±0.01	0.050±0.001	0.75±0.15
	AMPPNP[b]		207±39									
	ADP[b]		47.5±3.6									
	BDGA[c]		0.063±0.004									
Dissociation kinetics												
Wild-type	DMAG	0.25 mM									0.21 (0.14)[d]	
K106A		β-ME									0.50 (0.44)[d]	
S107A											0.12 (0.13)[d]	
Wild-type	DMAG	2 mM DTT									0.19 (0.02)[d]	
K106A											0.40 (0.06)[d]	
S107A											0.11 (0.02)[d]	
Anisotropy measurements												
Wild-type	BDGA[c]	None		480±129	414±30	536±180	473±119	489±114	422±138			
		DTT		111±9	42±3	30±2	27±5	24±3	29±4			
		TCEP		32±3	30±4	28±3	23±2	28±2	27±3			
K106A	BDGA[c]	DTT		171±32	166±32	125±28	120±20	118±20	117±13			
S107A	BDGA[c]	DTT		481±111	145±15	84±7	81±11	81±11	63±9			

[a] Numbers in parentheses indicate the values of k_{obs} after 24 h incubation.
[b] Data obtained by ITC.
[c] Data obtained by fluorescence anisotropy.
[d] Under the conditions used, the observed rate constant is not equal to the dissociation rate constant; however, the values are a good measure of the relative dissociation rates after 0 and 24 h incubation time.

and dissociation constants determined. These showed a time-dependent behaviour when protein and ligand were incubated for different lengths of time from time zero to 24 h (Figure 4(a)). As it has been suggested recently that the quinone moiety in ansamycins can be reduced slowly over time by reducing agents such as DTT, and that the resulting dihydroquinone species binds to Hsp90 more tightly,[15] we checked whether the differences in binding observed over time were simply due to the slow reduction of the ligand rather than a slow conformational change. The experiments were repeated in the absence of reducing agents and no change in binding affinity was observed under these conditions (Figure 4(b)). Further to this, samples incubated for 24 h in the absence of DTT, regained the time-dependent increase in binding affinity upon addition of reducing agent (data not shown). The data obtained indicates that the slow decrease in K_d seen over time is in fact due to a slow reduction of BDGA by DTT. To test this, we pre-incubated a sample of BDGA with 2 mM Tris(2-carboxyethyl)-phosphine (TCEP) before addition to the binding assay (Figure 4(c)). The reduced BDGA showed no time-dependent decrease in dissociation constant establishing that the tight binding of BDGA to Hsp90 is due to changes in the physico-chemical properties of the inhibitor and not to changes in Hsp90 conformation. The results obtained explain the difference in binding affinities observed between the fluorescence anisotropy and ITC experiments reported here and measured in the same assay buffer. In this case, the samples of protein and ligand are incubated for significantly longer (up to 24 h) in the fluorescence anisotropy experiments compared with minutes for the ITC experiments, allowing the slow reduction of the GA derivatives to the tighter binding dihydroquinone.

To confirm the results obtained using fluorescence anisotropy, the ITC experiment was repeated using a sample of DMAG that had been incubated overnight in ITC buffer containing 2 mM TCEP before measurement. Figure 4(d) shows that under these conditions there is a loss of the sigmodial binding isotherm observed in Figure 2. This

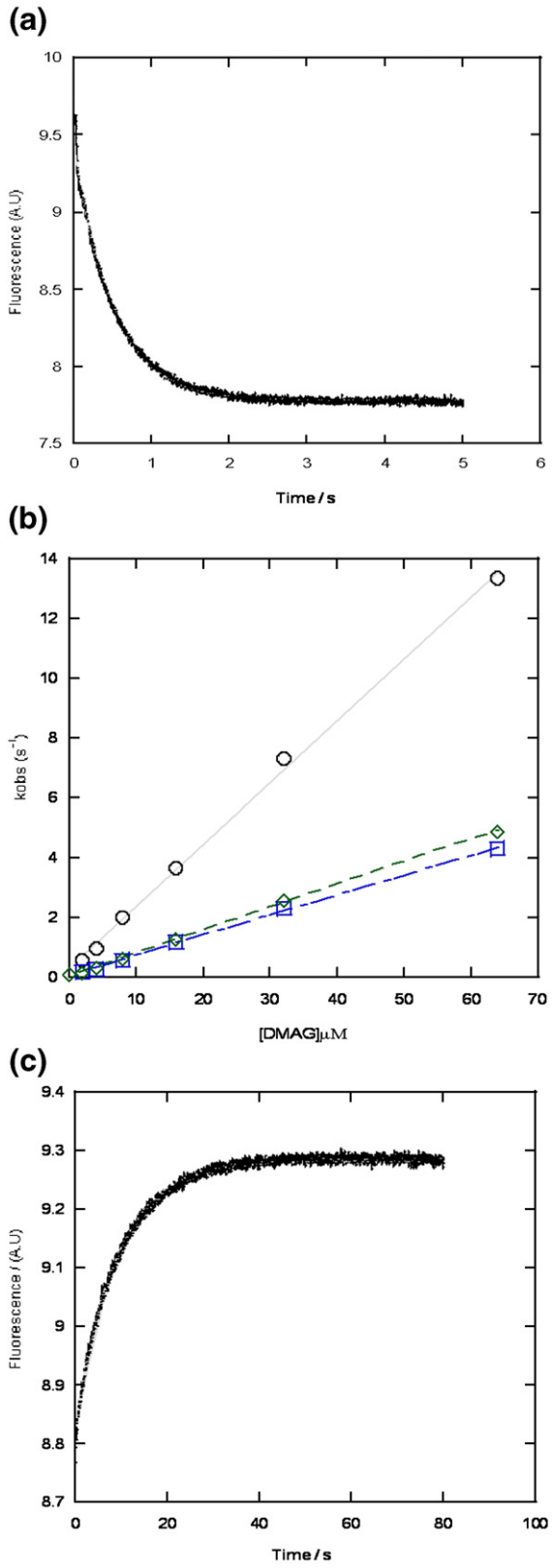
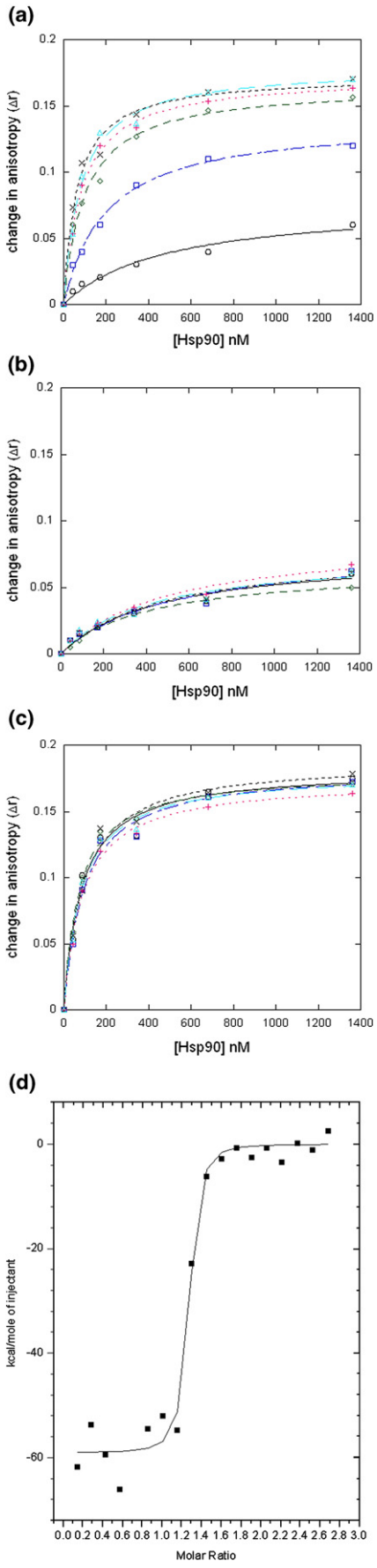


Figure 3. Association kinetics of DMAG binding to wild-type and mutant Hsp90. (a) The change in fluorescence after mixing 8 μM DMAG and 2 μM WT Hsp90 as measured by stopped-flow fluorimetry. Data were fit to a single-exponential equation. (b) The observed association rate constants for DMAG binding to wild-type (◇), K106A (○), S107A (□) as a function of ligand concentration. The data were fit by a linear least-squares algorithm. (c) Dissociation kinetics of DMAG–Hsp90 complexes. Change in fluorescence upon dilution of a pre-formed Hsp90–DMAG complex, measured by stopped-flow fluorimetry. Data were fit to a single-exponential equation. An Applied Photophysics Stopped-Flow Reaction Analyzer was used and data were acquired and analyzed using the Applied Photophysics Kinetic Workstation, version 4.099, supplied. Excitation was at 280 nm with a cut-off filter of 320 nm. Association kinetics were measured by mixing 20 μM Hsp90 rapidly with increasing concentrations of GA derivative or nucleotide in assay buffer at 37 °C and monitoring the change in fluorescence on binding. For dissociation experiments, a solution of a pre-incubated Hsp90–DMAG complex was diluted 1:50 (v/v) in assay buffer and the change in fluorescence was monitored over time.



indicates a significant increase in binding affinity following reduction of the ligand. A fit of the data gives a K_d of 2.2(±1.4) nM for the interaction of reduced DMAG with Hsp90, which is at the limit of accurate measurement for this technique (the low concentrations of protein and ligand needed to titrate within the low nanomolar range reduce

Figure 4. Binding of 10 nM BDGA to different concentrations of Hsp90 (0–1400 nM) monitored by fluorescence anisotropy. (a) Time-dependent binding of Hsp90 to BDGA in the presence of 2 mM DTT. (b) Time-dependent binding in the absence of reducing agents. (c) Pre-reduced BDGA binding to Hsp90 for (○) 10 minutes, (□) 1 h, (◇) 2 h, (×) 4 h, (+) 8 h, (△) 24 h. (d) Reduced DMAG binding to Hsp90 as measured by ITC. BODIPY-GA (BDGA) was synthesized as described.[18] Hsp90 was titrated into 10 nM BDGA in assay buffer (50 mM Tris–HCl (pH 7.4), 0.1 mg ml^{-1} bovine gamma-globulin, 2 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, and 0.1% (v/v) Triton X-100). Concentrations of ligand were low in order to (i) minimize the background signal, and (ii) be below the K_d. Molybdate was added as described.[17] ITC experiments in the absence and in the presence of 20 mM Na_2MoO_4 showed that molybdate does not affect DMAG binding significantly (data not shown). Triton and bovine gamma-globulin were added in order to reduce adhesion of ligand and protein, respectively, to the surfaces of the quartz cuvette, as is standard procedure for experiments of this type performed with low concentrations of materials. The fluorescence anisotropy (r) of samples of Hsp90 with various concentrations of BDGA and various incubation times (from 0 to 24 h) were measured in a Cary Eclipse fluorescence spectrophotometer, with a Peltier-controlled multicell holder (Varian); the excitation wavelength was 485 nm, and emission was monitored at 535 nm. Dissociation constants for BDGA were determined by fitting the anisotropy data to equation (1), which is the equation for the single-binding site model where the concentration of the Hsp90–ligand complex is directly proportional to the change in anisotropy (Δr):

$$\Delta r = \frac{\Delta r_{max}[Hsp90]}{K_d + [Hsp90]} \quad (1)$$

where K_d is the dissociation constant. Values for the dissociation rate constant, k_{off}, for BDGA were determined by displacement measurements in which a solution of 600 nM Hsp90 was incubated with 500 nM BDGA in the presence of 2 mM TCEP overnight. The samples were then diluted 100-fold into assay buffer containing 500 nM DMAG. The reaction was monitored by fluorescence anisotropy and the kinetic traces obtained were fit to a single-exponential function, to give the dissociation rate constant (data not shown). Isothermal titration calorimetry was performed using a MicroCal VP-ITC instrument (Microcal Inc., Northampton, MA USA). A 200 μl sample of 20 μM DMAG was injected into a 1.4 ml cell containing 1 μM wild-type Hsp90. Both Hsp90 and DMAG were incubated overnight in 50 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$ 20 mM KCl, 2 mM TCEP before the experiment, which was carried out in the same buffer conditions at 37 °C. Parallel experiments were carried out by injecting DMAG into assay buffer without Hsp90 to correct for the heat of dilution in the subsequent data analysis using the Origin software package (MicroCal Inc.). The concentrations of protein and DMAG were determined spectrophotometrically post injection.

the heat change that can be observed during the interaction).

The discrepancy between the low-affinity binding of GA derivatives to Hsp90 *in vitro* and the high-affinity anti-proliferative activity of these same compounds *in vivo,* in addition to the selectivity of inhibitors towards tumour cells, has been the subject of great debate over the last few years.[11,12] Here, we have investigated the mechanism of inhibitor binding to Hsp90 using the GA analogues DMAG and BDGA. Both the equilibrium binding, and the association and dissociation kinetics of DMAG to both wild-type human Hsp90 and the mutants K106A and S107A were undertaken. Whilst the equilibrium studies showed that there was a reduction in the affinity of DMAG for the K106A mutant, as expected from the crystal structure, we found that, contrary to reported data,[16] there was no appreciable difference in the affinity for the S107A mutant. Mutation of Ser113 had been shown to completely abrogate binding to GA in chicken Hsp90, despite the fact that it does not make any direct interactions with bound GA. Consequently, it had been suggested that this residue acted not in the binding of the compacted '*cis*' GA, but was involved in the docking of the extended *trans* molecule and aided in the conversion of *trans* to *cis* GA *via* a mechanism of keto-enol tautomerisation, in partnership with the lysine residue at position 112.[16] Whilst our equilibrium studies on the S107A mutant suggested it had little effect on the binding of DMAG, it was possible that, whilst the equilibrium dissociation constant was largely unaffected by this mutation, the association and dissociation rate constants changed. In particular, on the basis of the previous studies that hypothesized that Ser107 has an important role in the association reaction, one might expect a decreased association rate for the S107A mutant.[16] For wild-type Hsp90 and both mutants, the K_d values obtained from the kinetic rate constants agreed well with equilibrium binding constants, suggesting that there is no second step in the binding of DMAG to Hsp90. S107A bound to DMAG with k_{on} and k_{off} values of 6.7×10^4 M^{-1} s^{-1} and 0.050 s^{-1}, respectively, which agreed well with the values obtained for wild-type. Taken together, these results suggest that GA derivatives bind to Hsp90 in a single step and that the serine residue at position 107 is not involved in the binding.

As expected, the lysine mutant had a protracted off rate in comparison to wild-type Hsp90. However, and somewhat unexpectedly, the association rate constant for this mutant was significantly higher than the wild-type value. The increase in k_{on} for this mutant could be due to unfavourable electrostatic interactions between the positively charged tertiary amine of DMAG and the positively charged lysine residue in wild-type Hsp90. Removal of this charged lysine on mutation to alanine would remove these interactions and facilitate faster association of the ligand. The increased association rate seen in the K106A mutant explains why this mutant did not have a greater effect on the K_d of DMAG, and may explain why DMAG and 17-allylamino,17-demethoxygeldanamycin (17-AAG) appear to have a lower affinity for Hsp90 than GA, which lacks the tertiary amine group.[18]

It has been suggested that before binding to Hsp90, GA derivatives must first undergo a *trans-cis* isomerisation and flipping of the ansa ring. The *trans-cis* isomerisation has been calculated to be thermodynamically very unfavourable, and therefore unlikely to occur spontaneously in solution.[16] A recent study by Thepcatri *et al*.[19] used NMR analysis of molecular flexibility in solution (NAMFIS) to show that, while approximately 70% of the NAMFIS-derived solution structures of GA preferred a *trans* conformation, 30% exist in a *cis* configuration. However, a particular conformation that represented the bound form of GA (RMSD = 0.8 Å) represented 4% of the solution structures observed. While NAMFIS treatment has been known to erroneously overestimate the contribution of certain structures, the identification of a conformer resembling the protein-bound structure of GA indicates that GA binding to Hsp90 could involve the direct association of this species, in agreement with the data presented here.

It has been shown that the fluorescently labelled GA analogue BODIPY-GA (BDGA) bound to Hsp90 tightly in a time-dependent manner.[14] Here, we have studied the binding of BDGA to wild-type and mutant Hsp90. Our results, at first glance, appear to agree well with published data suggesting that Hsp90 initially binds to GA weakly and over time undergoes a structural change to a conformation that has a higher affinity. However, upon removal of reducing agents from our assay conditions, BDGA no longer bound to Hsp90 tightly in a time-dependent manner, with its binding affinity remaining in the low micromolar range over 24 h, consistent both with published data and data obtained in our ITC studies on DMAG (Figure 2). Further to this, BDGA pre-incubated in reducing conditions bound to Hsp90 with an affinity in the nanomolar range, and this affinity did not change over 24 h. ITC experiments using DMAG pre-incubated in buffer containing 2 mM TCEP also showed that a nanomolar affinity was obtained in the presence of reducing agents. In the absence of reducing agents, ΔH_{bind}, the enthalpy change associated with the binding of DMAG to Hsp90 was $-19.9(\pm0.3)$ kcal mol^{-1} whilst ΔS_{bind}, the entropy change on binding of DMAG to Hsp90, was -34.7 cal mol^{-1} K^{-1}. In the presence of reducing agents the ΔH_{bind} increased considerably to $-59.1(\pm1.6)$ kcal mol^{-1}, whilst ΔS_{bind} increased to -151 cal mol^{-1} K^{-1}. Thus, the binding of reduced DMAG to Hsp90 is associated with a large increase in the favourable change in enthalpy, presumably reflecting increased non-covalent interactions between Hsp90 and ligand. One potential source of the additional binding energy observed is the formation of new hydrogen bonds between the dihydroquinone hydroxyl groups and Asp54 (Figure 5). The increase in favourable enthalpy on binding is balanced by an increase in the loss of entropy on

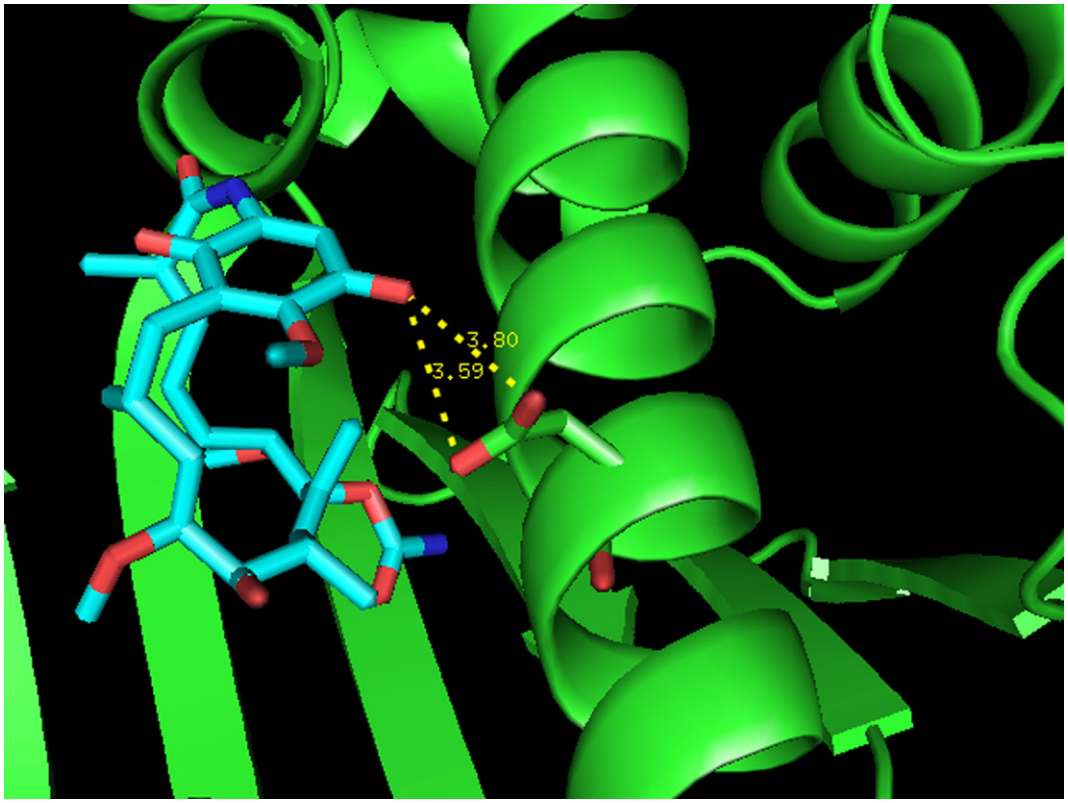


Figure 5. Interaction of DMAG with Asp54 showing potential hydrogen bonds that could be made with the dihydroquinone hydroxyl groups of reduced DMAG.

binding. This type of trade-off between enthalpy and entropy has been observed before,[21] and is thought to originate from the fact that tighter binding is associated with a higher degree of order in the bound state.

Our data provides further evidence that the reduction of the quinone moiety in GA derivatives increases the drug's affinity for Hsp90. The fact that these molecules can be reduced slowly over time in the presence of moderate reducing agents proves that previous reports suggesting time-dependent conformational changes in Hsp90 were actually measuring the slow reduction of the quinone moiety.[14]

The chemical modification of drugs *in vivo* is an important issue for pharmacokinetics and drug efficacy, this has been shown with many pharmacological agents. Both AZT (used to slow the progression of HIV) and acyclovir (used to treat herpes infections) are activated *in vivo* by phosphorylation,[22] while levodopa (used in the management of Parkinson's disease) is known to be converted by DOPA decarboxylase to active dopamine *in vivo*. Knowledge of the *in vivo* modifications required for drug efficacy can be used to design drugs that will have enhanced specificity and toxicity towards diseased cells.[23]

As stated in the study by Maroney and co-workers, many tumours have reduced blood flow, meaning that cancer cells often form a naturally reducing environment, rich in reductases, and it has been shown that certain reductases that are over-expressed in cancers cells targeted by Hsp90 inhibitors, such as the reductase DT diaphorase, can reduce GA *in vitro* from the quinone to the dihydroquinone.[15] The reduction of the quinone moiety in GA derivatives to dihydroquinone has now clearly been established as an important factor in determining the binding affinity of GA and its derivatives *in vivo* and this phenomenon is likely to contribute towards the selective targeting of cancer cells over normal cells for this class of compound. However, it has been shown that Hsp90 also appears to bind to GA with increased affinity in cell lysate obtained from normal cells,[15] and it is known that purine scaffold and other classes of inhibitors (which cannot be reduced) also appear to bind to Hsp90 in cancer cells with a higher affinity than normal cells.[12] Clearly, the reducing environment of many tumours does not completely solve the problem of increased specificity of Hsp90 inhibitors *in vivo*. Whether the reduction of the quinone moiety is the only factor affecting the increased binding of Hsp90 by GA derivatives *in vivo*, and the mechanism by which other Hsp90 inhibitors target Hsp90 *in vivo* remain unclear and continue to prove an interesting and challenging problem to researchers in the field.

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