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Blocking the chaperone kinome pathway: Mechanistic insights into a novel dual inhibition approach for supra-additive suppression of malignant tumors

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ABSTRACT

The chaperone Hsp90 is involved in regulating the stability and activation state of more than 200 'client' proteins and takes part in the cancer diseased states. The major clientele-protein kinases depend on Hsp90 for their proper folding and functioning. Cdc37, a kinase targeting co-chaperone of Hsp90, mediates the interactions between Hsp90 and protein kinases. Targeting of Cdc37 has the prospect of delivering predominantly kinase-selective molecular responses as compared to the current pharmacologic Hsp90 inhibitors. The present work reports a bio-computational study carried out with the aim of exploring the dual inhibition of Hsp90/Cdc37 chaperone/co-chaperone association complex by the naturally occurring drug candidates withaferin A and 17-DMAG along with their possible modes of action. Our molecular docking studies reveal that withaferin A in combination with 17-DMAG can act as potent chaperone system inhibitors. The structural and thermodynamic stability of the ligands' bound complex was also observed from molecular dynamics simulations in water. Our results suggest a novel tumor suppressive action mechanism of herbal ligands which can be looked forward for further clinical investigations for possible anticancer drug formulations.

1. Introduction

Molecular chaperones are required for the correct folding of many proteins inside cells. However, they also play a significant role in induction of cancer diseased states. The role of molecular chaperones in cancer was first characterized with regard to Hsp90 (heat shock protein 90) [1], which is a facilitator of oncogenesis that stabilizes a wide range of over expressed or mutated oncogenic proteins and permits their tumorigenic influence to arise [2,3]. Hsp90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells compared to their normal counterparts [4,5]. Hsp90 plays a key role in regulating the stability and activation state of more than 200 'client' proteins molecules [6]. Protein kinases, which represent the largest class of Hsp90 clientele, are known to be implicated in signal transduction, cell proliferation and survival, and many of them have been shown to cause cancer when deregulated.

However, Hsp90 does not directly bind to protein kinases but requires a series of co-chaperones to assemble into a super-chaperone complex for its function. These co-chaperones bind and leave the complex at various stages to regulate the chaperoning process. Arresting the chaperone cycle at these stages by targeting different co-chaperone/Hsp90 interactions seems to be quite a viable mode of inhibition and is likely to achieve similar consequences as that of Hsp90 direct inhibition with added favors of high specificity and reduced side effect profile.

Cdc37 (Cell division cycle protein 37), a co-chaperone of Hsp90 has been characterized as a kinase targeting subunit of the Hsp90 machinery [7], which mediates the interaction between Hsp90 and protein kinases. Cdc37 facilitates the maturation of these kinase clients by acting as an adaptor, loading these kinases onto the Hsp90 complex [2,8,9]. Combining Cdc37 silencing with the HSP90 inhibitor 17-AAG induced more extensive and sustained depletion of kinase clients and potentiated cell cycle arrest and apoptosis [10]. Thus targeting of Cdc37 has the prospect of delivering a different, predominantly kinase-selective and potentially more favorable set of molecular responses compared to current pharmacologic Hsp90 inhibitors. Moreover, the therapeutic blockade of Cdc37 could be used to enhance the effect of current Hsp90 inhibitors, as reported by other recent study [11]. These results support an essential role of Cdc37 in concert with Hsp90 in maintaining oncogenic protein kinase clients and endorse the therapeutic potential of targeting Cdc37/Hsp90 in cancer. Thus functional inhibition of both Hsp90 and Cdc37 in tumor cells would result in a supra-additive effect.

WA (withaferin A), a principle constituent of the plant *Withania somnifera*, has received much attention in recent years owing to its

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various pharmacological properties like antiinflammatory [12], antitumor [13,14], antibacterial [15] and many others. WA belongs to a family of steroidal lactones having withanolide skeleton as their basic structure. As is evident from the structure of WA (Fig. 1A) that it contains a lactone ring enclosed ester group, two conjugated ketone bonds and a three membered epoxy ring, all of which are quite susceptible to a nucleophilic attack. Most recently, it was shown to potentiate apoptosis of tumor cells by suppression of NF-κB activation [16–18]. Inhibition of HSF1 expression in cancer cells *in vivo* has also been reported to be induced by WA treatment [19]. Recently we have depicted the binding modes of action of WA on NF-κB signaling pathway [20] and proteasomal degradation pathway [21].

Natural product inhibitors of Hsp90 like radicicol, benzoquinone ansamycins like GA (geldanamycin) which are all based on competitive inhibition to ATP binding, cause the catalytic cycle of Hsp90 to arrest in the ADP-bound conformation, leading to the inactivation of chaperone activity and premature ubiquitination of client proteins [22,23]. In spite of potent anticancer activity exhibition of GA in pre-clinical *in vivo* studies, little clinical implication could be extracted out of it owing to its high hepatotoxicity observed in animal models [24]. This has led to the exploration of GA derivatives like 17-AAG (17-allylamino geldanamycin) and 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) having similar anticancer activities as to GA but with better toxicological properties [25,26]. 17-DMAG (Fig. 1B) which is a more soluble analog of 17-AAG merits 17-AAG owing to its high solubility, increased stability in solution and high oral bioavailability [27,28].

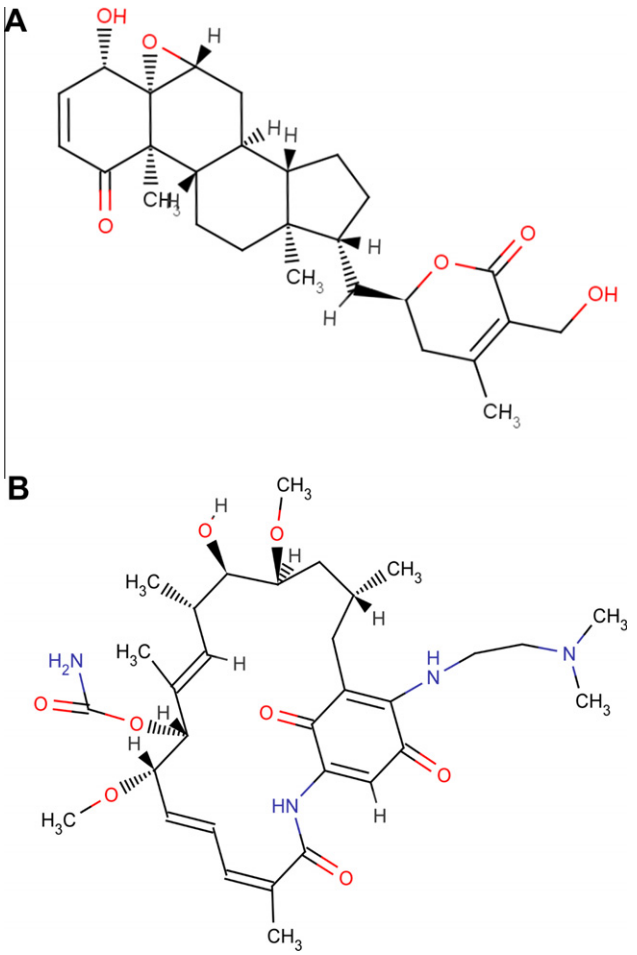

Fig. 1. Structures of ligands. (A) Structure of WA. (B) Structure of 17-DMAG.

In the present study, we report the dual inhibition of Hsp90/Cdc37 complex by 17-DMAG and WA as is revealed by our successive molecular docking analysis. The stability of the binding complex was also demonstrated by molecular dynamics simulations. Our analysis also elucidates the molecular mechanism of action of these proposed drugs on the associated chaperone/co-chaperone target.

2. Materials and methods

2.1. Ligands and receptors

The crystal structure of the Hsp90/Cdc37 association domain [PDB: 2K5B] was obtained from the Protein Data Bank (PDB) [29]. Before docking, the protein crystal structure was cleaned by removing the water molecules. H-atoms were added to these target proteins for correct ionization and tautomeric states of amino acid residues. The modified structure so obtained was used for all the dual docking studies. The ligand molecules WA (PubChem:265237) and 17-DMAG (PubChem:5288674) were retrieved from NCBI–PubChem Compound database [30]. The energies of the ligand molecules and receptor were minimized in steepest descent and conjugate gradient methods using AMBER v.11 [31].

2.2. Structural aspects of Hsp90–Cdc37 complex

Hsp90 is composed of three domains: an N-terminal domain of ~25 kDa, a middle domain of ~35 kDa, and a C-terminal domain of ~10 kDa [32]. The ATP-binding site of Hsp90 is located in the N-terminal domain. The 44.5-kDa Cdc37 protein can be dissected into three domains [33,34]: an N-terminal domain (residues 1–127 ($Cdc37_N$), 15.5 kDa), a middle domain (residues 147–276 ($Cdc37_M$), 16 kDa), and a C-terminal domain (residues 283–378 ($Cdc37_C$), 10.5 kDa). The middle domain $Cdc37_M$ is highly resistant to proteolytic digestion and was found to be the most stable domain of Cdc37 [35]. Cdc37 associates with the N-terminal portion of protein kinases [36,37]. The 20 residue client binding site of Cdc37 (181–200) and the glycine rich loop in the N-terminal portion of the protein kinases are both necessary for physical interaction between the two proteins [36,38]. The middle segment of Cdc37 has also been shown to interact with the N-terminal ATP-binding site domain of HSP90 [39].

2.3. Ligand docking

AutoDock 4.0 suite was used as molecular-docking tool in order to carry out the docking simulations [40]. Rigid roots were assigned to the ligands and thus five and eight bonds were made "active" or rotatable for the ligands WA and 17-DMAG, respectively. The modified structures so obtained: crystal structure of Hsp90/Cdc37 complex and the structures of ligands WA and 17-DMAG accounting the flexibility of its bonds, were converted to PDBQT format in ADT, as required in AutoDock calculations. The Lamarckian Genetic Algorithm (GA) was used with a population size of 150 dockings. The highly stable structure out of all the docked structures was chosen for carrying out second docking with 17-DMAG. The grid size for specifying the search space for first ligand WA was set at 40 × 70 × 50 with a default grid point spacing of 0.375 Å. The grid size for second ligand 17-DMAG was set at 40 × 40 × 40. The results are clustered into bins of similar conformations according to the cluster root mean square deviation (rmsd) and orientation.

2.4. Selection and representation of docking modes

AutoDock reports the best docking solution (lowest docked free energy) for each GA run and also performs a cluster analysis in

which the total number of clusters and the rank of each docking mode (cluster rank) are reported. Docking modes were selected on the basis of two criteria: extent of ligands' associations with the key residues of the receptor and the thermodynamic stability of the docked complex so obtained. For a 10 GA run there would be up to 10 total docking modes from which the lowest energy-docking mode was chosen that met the above two criteria. All the AutoDock docking runs were performed on Intel Core 2 Duo P8400 CPU at 2.26 GHz of Sony origin, with 3 GB DDR RAM. AutoDock 4.0 was compiled and run under Windows VISTA operating system.

2.5. MD simulations in water

The AMBER v.11 package [41] was used to prepare the protein and the ligand files as well as for the Molecular Dynamics (MD) simulations. The binding complexes of Hsp90/Cdc37/WA and Hsp90/Cdc37/WA/17-DMAG obtained using AutoDock, and the un-docked Hsp90/Cdc37 association protein simulated in this study were neutralized by adding appropriate number of sodium counter-ions and were solvated in a octahedron box of TIP4PEW water with a 10 Å distance between the protein surface and the box boundary [42]. The partial atomic charges for the ligands were obtained after optimization at the Hartree–Fock level with 6-31G* basis set and subsequent single-point calculation of the electrostatic potential to which the charge were fitted using RESP procedure [43,44]. Force field parameters of the ligands were assigned based on the atom types of the force field model developed by Cornell et al. [31].

The binding complex was effected with a 750 step minimization using SANDER module of AMBER in the steepest descent followed by a 250 step minimization in conjugate gradient. Then the system was equilibrated beginning with the protein atom restrained simulations having 200 ps equilibration dynamics of the solvent molecules at 300 K. Next step involved the equilibration of the solute molecules with a fixed configuration of the solvent molecules in which the system was slowly heated from $T = 0$ to 300 K in 60 intervals each involving heating for a 5 K increase in 2.5 ps followed by an equal time duration equilibration step. The entire system was then equilibrated at 300 K for 200 ps before a sufficiently long MD simulation (for 2.7 ns) at room temperature. The MD simulations were performed with a periodic boundary condition in the NPT ensemble at $T = 298.15$ K with Berendsen temperature coupling [45] and constant pressure $P = 1$ atm with isotropic molecule-based scaling. We used a time step of 2 fs and a non-bond interaction cutoff radius of 10 Å. MD simulations were performed on a 320 processors SUN Microsystems clusters at Supercomputing Facility (SCFBio) at IIT Delhi.

3. Results and discussion

3.1. Docking of WA into Hsp90/Cdc37 association complex

One possible mode of action which has been proposed here for WA to act as an anticancer agent is by suppression of the kinase binding activity of Cdc37 which depends on certain key residues (181–200) of Cdc37, which are essentially responsible for binding to the client kinases. Using binding pocket analysis, a cleft encompassing these residues was obtained as one of the putative binding site. As evident from the docking of WA into Cdc37 (Fig. 2A), WA is trapped inside this protein cleft. Fig. 2B shows the ligand occupying the key residues' enclosed cavity of the receptor, being represented as a mesh surface. As AutoDock reports the best docking solution for each GA run and also performs a cluster analysis in which the total number of clusters and the rank of each docking mode (cluster rank) is reported, in 3 out of 10 docked

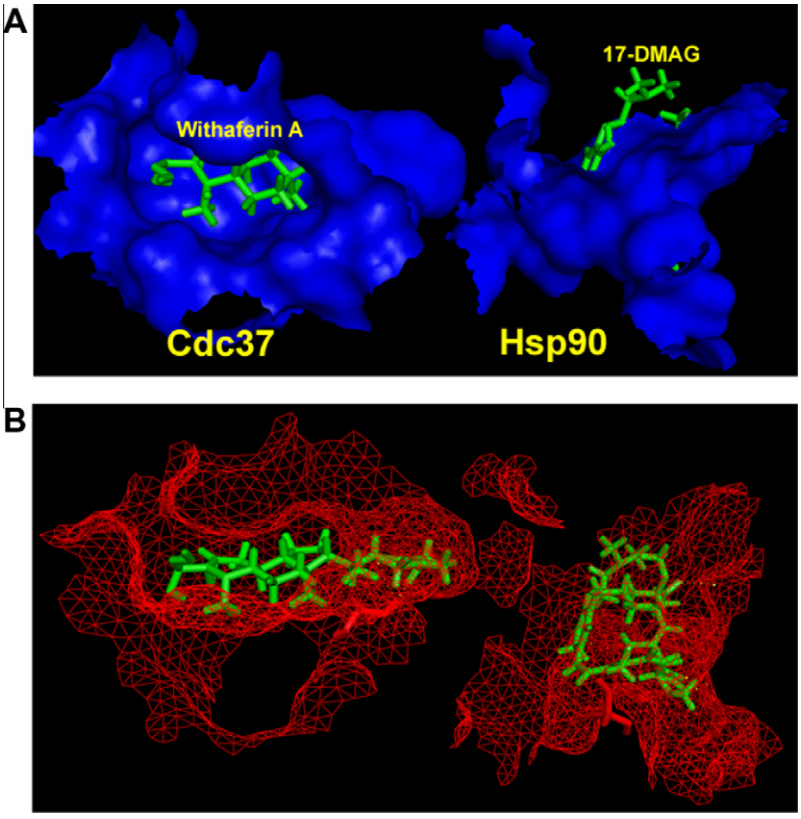

Fig. 2. Docking representations of WA and 17-DMAG into Cdc37/Hsp90 association complex. (A) Docking of WA into the cavities of the complex. (B) Docked ligands being trapped inside the pockets of the receptor subunit represented as mesh.

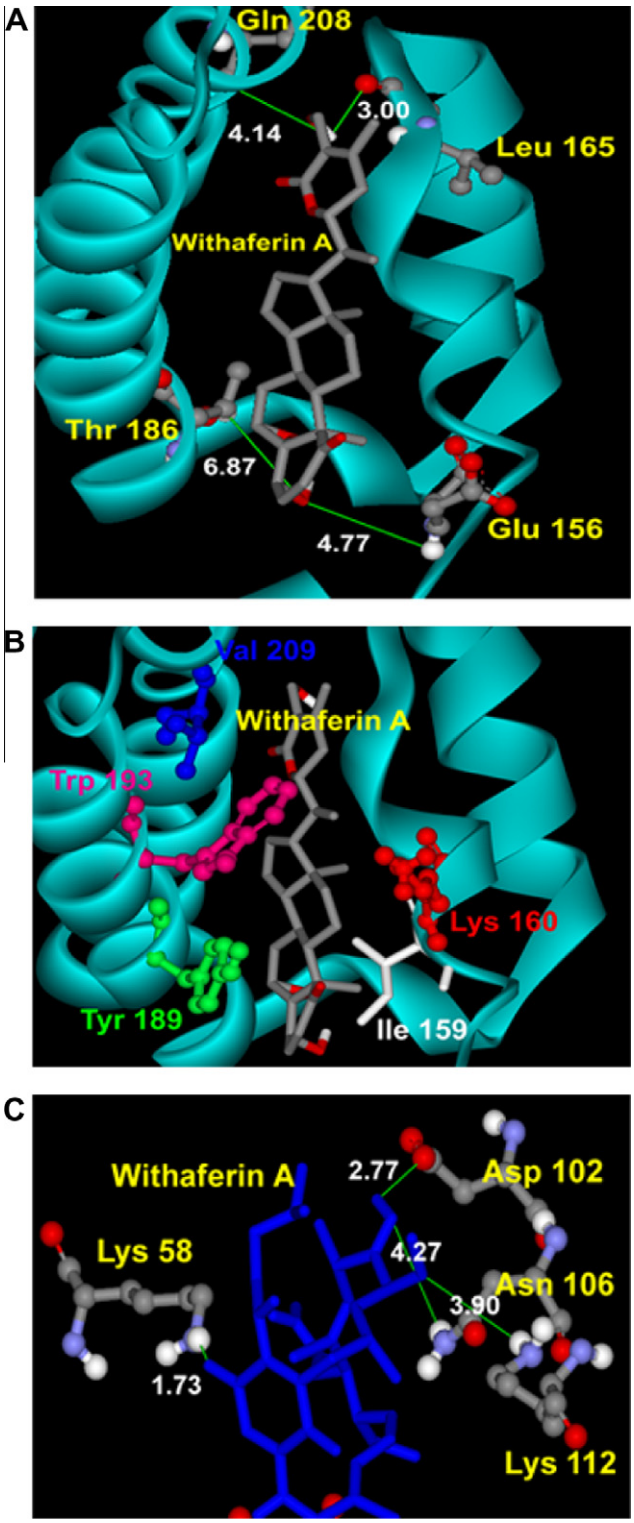

Fig. 3. Interactions present in the docked structures. (A) Docked WA forming H-bond interactions with key residues of Cdc37 in the Hsp90/Cdc37/WA/Cdc37 combinatorial docked complex. (B) van der Waals interactions of docked WA with key residues of Cdc37 in the Hsp90/Cdc37/WA/Cdc37 combinatorial docked complex. (C) 17-DMAG forming crucial H-bond interactions with the key residues of Hsp90 in the Hsp90/Cdc37/WA/Cdc37 combinatorial docked complex.

conformations (30% clustering frequency) obtained by the clustering analysis at 2.0 Å, the polar groups of WA are found in close contacts to the polar groups of the receptor (Fig. 3A). The various properties of the docked conformation are shown in Table 1. The

Table 1
Energies obtained after successive dockings.

Property	WA docked into Hsp90/Cdc37	17-DMAG docked into Hsp90/Cdc37/WA
Binding energy	−6.83 kcal/mol	−5.09 kcal/mol
Inhibition constant	9.88 μM	186.74 μM
Intermolecular energy	−6.98 kcal/mol	−6.20 kcal/mol
Total internal energy	−1.22 kcal/mol	−1.08 kcal/mol

binding energies of the conformations of this cluster range from −6.83 to −6.69 kcal/mol. The highest binding energy was obtained for a conformation in which the extent of H-bonding and van der Waals interactions (Fig. 3B) of the ligand with the receptor was maximum. Binding of WA to this cleft would result in providing hinderance to the kinase binding ability of Cdc37 arresting the chaperone-kinase binding thus obscuring kinases from their nefarious maturation.

3.2. Docking of 17-DMAG into docked Hsp90/Cdc37/WA complex

Binding energy of −5.09 kcal/mol was obtained from docking of 17-DMAG into the single docked Hsp90/Cdc37/WA complex. The various properties listed in Table 1 provide sufficient results in order to support the ongoing mechanism of dual inhibition of chaperone/co-chaperone complex by the two ligands. Docked 17-DMAG positions itself into the binding pocket of the Hsp90 receptor. Moreover the ligand occupies a conformation as required to facilitate the formation of a number of H-bond interactions with a clustering frequency of 60% (Table 2). As shown in Fig. 3C, C-11 hydroxyl group of 17-DMAG is involved in H-bonding with side chain acid group of Asp 102 and also with side chain amide group of Asn 102. Methoxy group at C-12 also forms H-bond with side chain amino group of Lys 112. The C-21 ring carbonyl group forms H-bond with Lys 58 and C-1 amide carbonyl of ansa ring with Asp 51. Binding of 17-DMAG to Hsp90 would cause its inhibition thus resulting in degradation of important signaling proteins involved in cell proliferation, cell cycle regulation and apoptosis.

3.3. MD simulations in water

The association complex Hsp90/Cdc37, the single docked protein–drug binding complex Hsp90/Cdc37/WA with the binding energy of −6.83 kcal/mol and the dual docked protein–drug binding complex Hsp90/Cdc37/WA/17-DMAG with the binding energy of −5.09 kcal/mol were used for carrying out MD simulations. After MD simulations, we calculated RMSDs between Cα trajectory of the association complex and Cα of its PDB crystal structure recorded every 2.5 ps. The RMSDs for the trajectory of the association protein complexed with WA and with both the ligands WA/17-DMAG were also calculated using their initial docked structures as references. The results in Fig. 4A show that the RMSDs of the trajectory of the single docked complexes were always less than 2.5 Å for the entire simulation suggesting the stability of the system. The adherence of the total energy trajectories to more or less constant values for the association complex and for both the docked complexes were observed during the entire simulation length (Fig. 4B), with the energy values of the dual docked complex (blue) much lowered than that of the single docked complex (green) and of the native protein (red), indicating thermodynamic stability of the combinatorial complex. The simulation length used in this study was long enough to allow rearrangement of side chains of the native as well as the drug complexed proteins to find their most stable binding mode. Thus the present MD simulations along with the molecular docking experiments made clear the dynamic structural stability of the chaperone assembly in the combinatorial

Table 2
Clustering results obtained from docking of 17-DMAG into Hsp90/Cdc37/WA complex.

Receptor	No. of AutoDock clusters[a,b]	Cluster rank[b]	No. of structures in the cluster	Lowest binding energy of cluster	Energy range within cluster
Hsp90/Cdc37 in complex with WA	5 (10)	1	6	−5.09	−5.09 to −3.67
		2	1	−2.27	−13.58 to −10.11
		3	1	−1.77	−1.77
		4	1	−1.33	−1.33
		5	1	0.18	0.18

[a] Number of GA runs are shown in parentheses.
[b] Clustering is done with RMS tolerance of 2.0 Å.

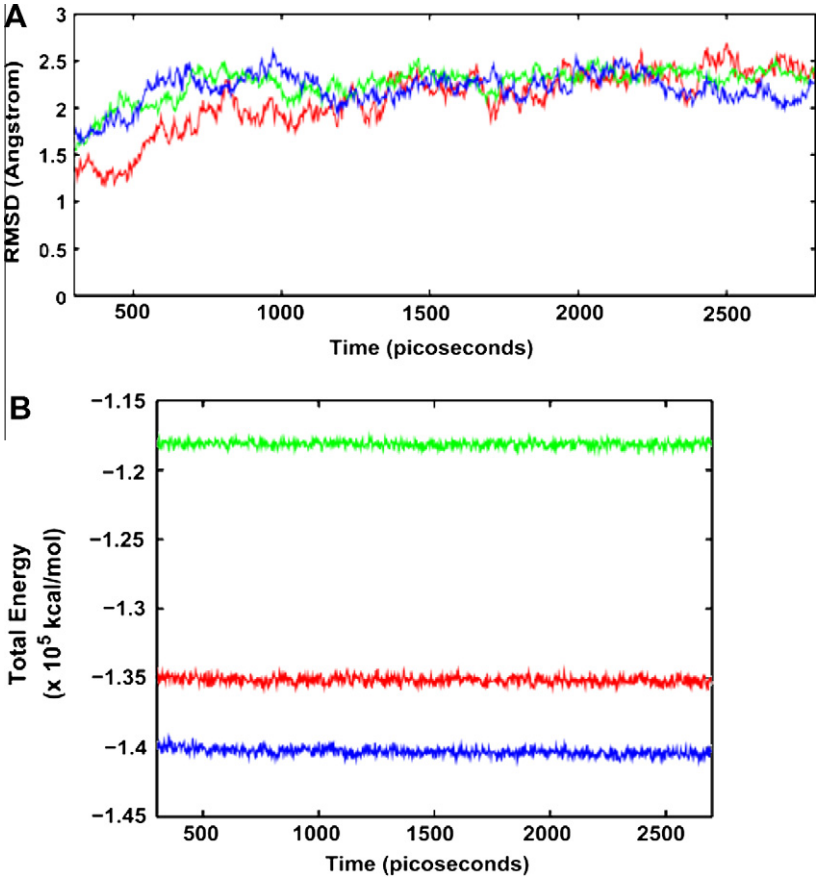

Fig. 4. (A) Plot of root mean square deviation (RMSD) of Cα of Hsp90/Cdc37 (protein) and Hsp90/Cdc37/WA (complex). RMSDs were calculated using the initial structures as templates. For protein (red) the reference is the modeled structure and for complex (blue) the reference is the initial model. The trajectories were captured every 1 ps until the simulation time reached 2700 ps. (B) Plot of total energy of h20S and h20S/WA (complex). The energy trajectories of both the protein (red) and the complex (blue) are stable over the entire length of simulation time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complex with the drugs WA and 17-DMAG, together with the inhibitory mechanism.

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