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MDM2 Case Study: Computational Protocol Utilizing Protein Flexibility Improves Ligand Binding Mode Predictions

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ABSTRACT

Recovery of the P53 tumor suppressor pathway via small molecule inhibitors of onco-protein MDM2 highlights the critical role of computational methodologies in targeted cancer therapies. Molecular docking programs in particular, have become an essential tool in computer-aided drug design by providing a quantitative ranking of predicted binding geometries of small ligands to macro-molecular targets based on binding free energy, and allows for the screening of large chemical libraries in search of lead compounds for cancer therapeutics. In this study, we found improved ligand binding mode predictions of small medicinal compounds to MDM2 using AutoDock and AutoDock Vina while adopting a rigid ligand/flexible receptor protocol. Crystal structures representing small molecule inhibitors bound to MDM2 were selected from the protein data bank and a total of 12 rotatable bonds was supplied to each complex and distributed systematically between the ligand and binding site residues. A docking run was performed for each configuration and evaluated in terms of the top ranked binding free energy and corresponding RMSD from the experimentally known binding site. Results show lowest RMSD values coincide with the ligand having no or few rotatable bonds, while the protein retained all, or the majority of flexibility. Further, we found AutoDock Vina mirrored these results, while requiring substantially less computational time. This study suggests the future implementation of a rigid ligand/flexible receptor protocol may improve accuracy of high throughput screenings of potential cancer drugs targeting the MDM2 protein, while maintaining manageable computational costs. The continued evaluation and optimization of these programs complimented by advanced computer architecture will aide in reducing the cost of cancer drug development, as well as foster new insights into bio-molecular binding processes.

Keywords: MDM2, Molecular Docking, AutoDock, AutoDock Vina, Molecular Dynamics, Drug Discovery

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1. INTRODUCTION

P53 is a tumor suppressor protein found in the nucleus of cells, which functions to respond to cellular stress by mediating cell-cycle arrest, senescence, or apoptosis in response to DNA damage, oncogene activation, and hypoxia [1,2]. Inactivation of the P53 pathway is found in the majority of human cancers and is facilitated by mutation or deletion of the TP53 gene or damage to cellular regulatory mechanisms [3-5]. The primary regulator of P53 is murine double minute 2 (MDM2), an E3 ubiquitan ligase protein, which binds to P53 marking it for degradation. In damaged cells, over-expression of MDM2 results in reduced levels of P53, initiating the onset of oncogenesis [6,7]. Chemotherapies attempt to block this interaction and recover tumor suppression activity by introducing small non-peptide molecules designed to target and bind to the P53 binding domain of MDM2 [6-8]. Several small molecule inhibitors have been designed from lead compounds discovered via the structure-based virtual screening of chemical libraries performed by docking programs and many have entered and completed Phase 1 cancer drug clinical trials [9,10]. These high throughput screenings (HTS) evaluate thousands of small molecules and are a cost effective approach designed to rely on fast, accurate predictions, intended to isolate a small number of promising leads as future cancer therapeutics [11,12].

1.1 Molecular Docking: A Computational Methodology Aiding Drug Design

Molecular docking programs represent a critical tool in the early stages of structure-based drug design, (SBDD) while providing important insights into molecular binding processes [13,14]. The focus on MDM2 as a target for cancer therapeutic research and subsequent literature underscores the importance of docking programs such as AutoDock and AutoDock Vina, (henceforth referred to as Vina) for the quick and accurate screening of cancer drug candidates [14-16]. These programs conduct virtual screenings of small molecules from chemical libraries attempting to manage and resource the vast chemical space of all possible compounds available to be optimized as future cancer drugs [17]. HTS can exclude or include available compounds *in silico*, as drug leads based on desired binding geometry; often referred to as “binding pose” or “binding mode”, and binding free energy, a quantitative measure of the binding affinity between molecules. [18]. Due to the incredible complexity inherent in simulating the dynamics of the molecular binding process, docking programs introduced a time-independent strategy based on chemical potentials rather than the force calculations associated with classical molecular dynamics (MD), which adopt time dependent Newtonian physics [19,20]. In practice, a balance must be struck between computational time and accuracy. Therefore, docking programs rely on energy evaluations based on assumptions, estimates and empirical knowledge while estimating, rather than calculating, binding free energy [21].

The specific location and binding mode, along with a strong corresponding binding affinity are the two components of a successful docking [22]. The binding affinity is determined by the estimated binding free energy calculated from evaluations based on different molecular interactions and reflects the strength of the non-covalent physical binding. [13]. Due to the reliance on estimates and assumptions inherent in the free energy calculations, the binding free energies are generally viewed as un-reliable as a true measure of the free energy. Even when a particular binding mode can be determined, the binding affinity is in doubt due to the complex interactions estimated by the semi-empirical scoring functions often employed by docking programs using simplified free energy models. This problem arises when the experimental binding free energies determined from dissociation constants are quantitatively different from the estimates used for docking experiments [23]. However, docking methodologies in SBDD are widely used as they provide a complimentary technique for the discovery and optimization of lead compounds targeting proteins in addition to DNA intercalates and minor groove binders, designed to disrupt cancerous cell replication [24].

1.2 Overview of AutoDock and Vina

AutoDock’s efficacy and limitations have been well documented due to wide use in academic and commercial environments. AutoDock has been shown to provide fast and accurate predictions within 2 angstroms of the experimentally known binding site for ligands with up to 6 rotatable bonds. However, as the ligands’ rotatable bonds increase, performance decreases largely due to the exponential increase of possible conformational states [16, 25-27]. This restriction has been a notable difficulty pertaining to protein-ligand docking and has led to the use of a rigid receptor protocol as standard methodology because of the computational challenges posed by incorporating protein flexibility [28,29]. This

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methodology fails to account for side-chain residue movement at binding site interfaces, resulting in a less reliable prediction of the ligands' docked binding mode [13,30]. We know the accurate, computational simulation of protein conformational changes is critical to improved docking studies because it accounts for changes affecting the final binding geometry [19]. It has been shown that, when only a rigid receptor conformation is considered, docking studies predict incorrect binding poses for about 50–70% of all ligands [20]. In response, AutoDock introduced a feature that provided incorporation of protein flexibility, which is intended to account for a portion of the conformational changes of the protein upon binding. However, the flexibility of the protein and more importantly, the binding site residues, are still subject to the limits imposed by the number of rotatable bonds; therefore, this feature is limited to the ligand and protein having a total of about 10 rotatable bonds. While invoking AutoDock's side-chain flexibility feature accounts for some protein movement, the additional conformational search space associated with a flexible ligand and protein can reduce accuracy and increase computational costs [31].

Vina, a faster, more accurate alternative, was released in 2010, and was able to improve accuracy, while drastically reducing computation time through effective computer architecture and incorporating a "machine learning" approach for the scoring function [32]. Vina was tested using the same complexes evaluated during the development of AutoDock 4 and results show a marked improvement in terms of ligand binding mode accuracy. Vina's combination of speed and accuracy has made it an ideal program for HTS and has been used in several research studies and novel docking approaches [12,33]. Although AutoDock 4 and Vina share similarities in the use of an empirically weighted scoring function and global search optimization algorithm, they differ in their local search strategy and scoring function parameters [32].

1.3 Improving Ligand Binding Mode Predictions

As aforementioned, the two most significant results from a docking experiment include the binding free energy of a particular bound complex and the ligand binding mode prediction. The focus of this research is improving the ligand binding mode predictions of AutoDock and Vina through selective flexibility of the ligand and receptor by invoking AutoDock's feature of protein residue flexibility, while utilizing the speed and accuracy of Vina to reduce computational time. Few studies have highlighted AutoDock's protein flexibility feature, and no study has been found regarding results from adjusting ligand flexibility. Our re-docking study revealed improved binding mode predictions of small medicinal compounds to MDM2 based on RMSD values from the known binding site. Analysis of these results was supplemented by a classical MD simulation performed by the Nanoscale Molecular Dynamics program (NAMD) [34]. MD simulation programs, such as NAMD, utilize classical Newtonian physics to study the time dependent structure, dynamics, and thermodynamics of biological molecules. The microscopic properties of atomic positions and velocities can be translated into macroscopic quantities including temperature, pressure and volume using statistical mechanics. This enables determination of movement associated with selected binding site residues of the target protein. [35]. The docking and MD results from this study highlights the importance of modeling protein flexibility for the determination of accurate binding mode predictions of small molecules to MDM2 and may be especially useful for HTS of potential cancer drugs focusing on different protein and DNA targets. Cancer therapeutic research relies on a critical understanding of bio-molecular interactions enhanced by the effectiveness of computational techniques such as molecular docking [22]. Particular attention to validation and protocol inquires will ensure proper use and enable further modifications.

2. METHODS AND MATERIALS

Standard docking experiments employ a rigid receptor/flexible ligand protocol while exploring conformational space within a specified grid box designated by the user. Results are assessed according to the binding free energy, which represents a measure of how securely the small molecule is attached to the desired area of a target molecule. The binding mode is then analyzed to ensure the small molecule's correct location and geometry accompanies the top ranked binding energy. A successful re-docking will be within 2 angstroms of the experimentally known binding site and correspond to one of the top ranked binding energies [32,36,37]. In short, the sum of the energy of ligand and receptor separately is greater than the total energy when bound together. The difference is the binding free energy. A higher negative energy indicates a deeper potential energy well, a more stable complex, and more likely binding mode [38].

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For this study, only the top ranked binding energy and corresponding RMSD from the known binding site was considered as a data point.

2.1 Experimental Details

A set of four structures, representing small molecule inhibitors in complex with MDM2 was retrieved from the protein data bank (PDB). PDB codes: 4JRG (12), 3LBK (5), 4IPF (10) and 4ZYI (9). The number of inherent rotatable bonds in each ligand is given in parenthesis. For each complex, our protocol systematically distributed a total of 12 rotatable bonds between the ligand and receptor until all combinations were tested beginning with 0 flexible bonds for the ligand and 12 for the receptor, and then 1 flexible bond for the ligand and 11 for the receptor and so on, using the notation (0,12) and (1,11) respectively. When the maximum number of rotatable bonds was reached inherent in each ligand, the remainder was transferred to the protein. Docking parameters for all calculations using AutoDock 4.2 were adjusted to 100 runs with 2 x 10^7 energy evaluations and a grid box size of 60Ǻ 62Ǻ 62Ǻ centered on the ligand with .375 Å grid spacing. The grid box for Vina 1.1.2 was set to 27Ǻ 27Ǻ 27Ǻ centered on the ligand with a 1 Å grid spacing and the exhaustiveness was set to 12. All other settings for both programs were kept at default parameters.

All structures were retrieved from the PDB and initially prepared for docking using Chimera software [39] . The ligand was separated from the protein and a short energy minimization was applied to each structure for a duration of 10 steps. Hydrogen atoms were added, water molecules removed and Gasteiger charges were added to the ligand and protein. The necessary files for docking were prepared in AutoDock tools (ADT). When files are imported into ADT, they are checked for polar hydrogens, water molecules and proper charges. The rotatable bonds of the ligand were altered using the 'choose torsions' option. Here, the initially flexible bonds of the ligand can be adjusted for docking and saved. A flexible residue file was also created for the rotatable bonds of the selected protein binding site residues in addition to a separate rigid protein file.

AutoDock results are ranked according to the highest negative binding free energies and corresponding RMSD values from the experimentally determined binding site. Vina presents the binding energies with the top ranked binding free energy always corresponding to a 0 RMSD. The subsequent RMSD values are in relation to this top ranked pose. Determining if Vina and AutoDock can converge on a similar binding mode can be accomplished utilizing visualization software, which can directly compare the experimentally known structure to both programs best prediction. AutoDock and Vina share functional commonalities including the global optimization of the scoring function, pre-calculation of grid maps, and the pre-calculation of distant dependent pair-wise energetics between each atom type. However, they employ a different scoring function and algorithms to obtain binding free energies, and should be considered different programs [32]. Conveniently, both programs utilize the same ligand, receptor, and flexible residue files. This allows for a seamless transition between the two programs when the input files are prepared. These files are included as supplemental materials in addition to the docking parameter files and grid parameter files for AutoDock, and the conf.txt, log, and output files for Vina (S1).

3. RESULTS AND DISCUSSION

The MDM2 protein is a current target for cancer drug development in the form of small molecule inhibitors designed to firmly attach to its P53 binding domain, thus blocking P53/MDM2 interaction. This domain contains 9 or 10 binding site residues depending on the crystal structure, each with between 1 and 4 rotatable bonds. We sought to improve binding mode predictions using the popular docking programs AutoDock and Vina through the selective flexibility of both ligand and binding site residues using four crystallized structures obtained from the PDB. The docking results indicate best binding mode predictions correspond to ligand rigidity and protein flexibility for all structures tested using either program. Further, configurations of complete ligand rigidity (0,12), produced smaller RMSD values compared to the standard protocol for all structures. Surprisingly, supplying additional rotatable bonds well past AutoDock's usual accuracy threshold improves binding site predictions when this additional flexibility is transferred to the protein. The PDB structure 3LBK represents a small molecule in complex with MDM2 that contains only 5 inherent rotatable bonds, but AutoDock's most likely docked pose has it 2.20 Ǻ from the experimentally known site using the standard protocol in contrast to .51 Ǻ, when 12 rotatable bonds are supplied to selected binding site residues (figure 1). A snapshot of each of

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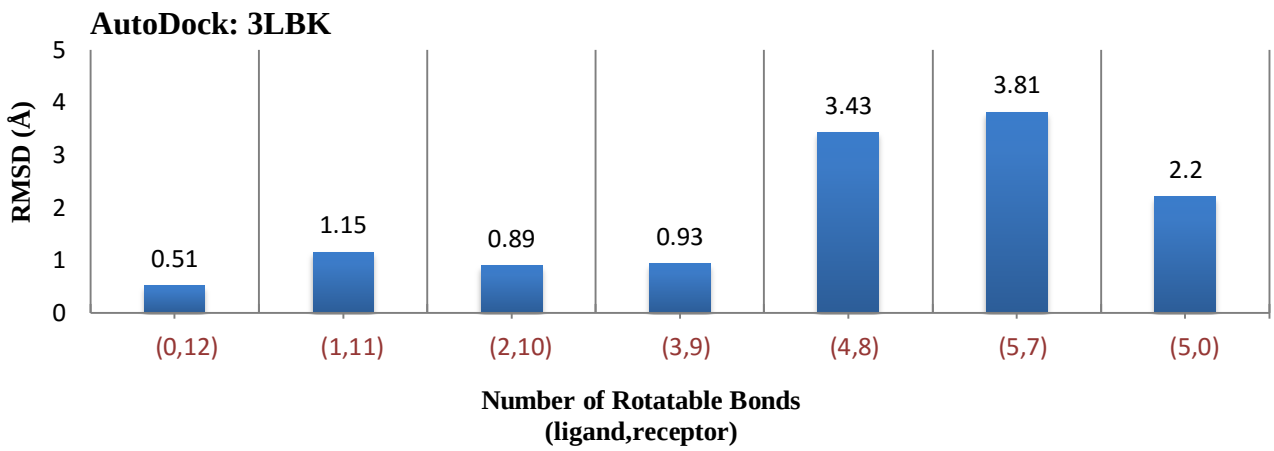

Fig. (1). RMSD values corresponding to the top ranked binding energies for all configurations indicate a total rigid ligand (0,12) has the lowest RMSD value of .51 Å. As the number of rotatable bonds become more evenly distributed, binding mode accuracy declines.

these two binding modes demonstrates the contrast between AutoDock's best prediction and the experimentally determined binding geometry (figures 2 and 3). We can see the juxtaposition of predicted and experimental geometries of configuration (5,0) representing the standard protocol and (0,12), the rigid ligand docking. The geometry and proximity of the standard protocol docking is not nearly as precise as (0,12), shown by the 2.20 Å RMSD as compared to .51 Å. The ligand bound to MDM2 in structure 4JRG contains 12 rotatable bonds, which is well above AutoDock's validated limit for a successful docking. Using the standard protocol (12,0), AutoDock's best prediction is 2.83 Å from the experimentally determined binding mode, while a rigid ligand protocol (0,12), yields a prediction within .58 Å (figure 4). Other structures tested, 4IPF and 4ZYI, also show improved binding mode predictions with lower RMSD values for (0,12) configurations as compared to standard protocol. Complete results

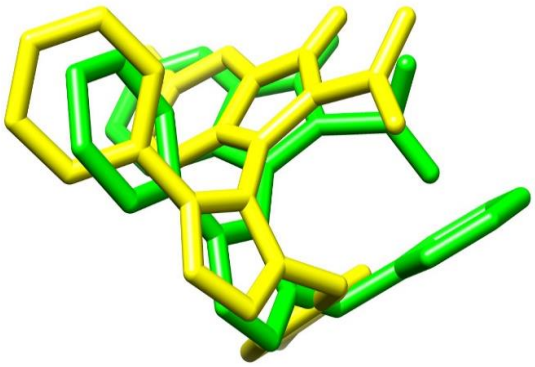

Fig (2). The standard protocol docking pose for 3LBK (5,0) shown in green using AutoDock with an RMSD of 2.20 Å. In contrast with the experimentally determined structure (yellow).

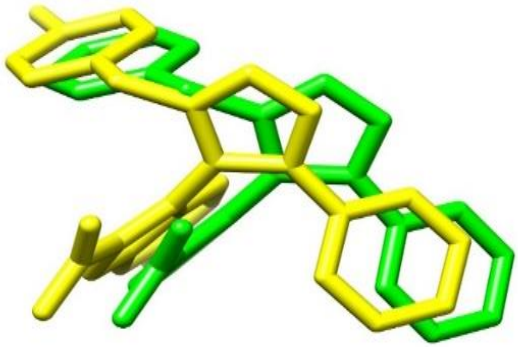

Fig (3). 3LBK rigid ligand configuration (0,12) using AutoDock with RMSD of .51 Å. Shown in green. In contrast to the experimentally known structure (yellow).

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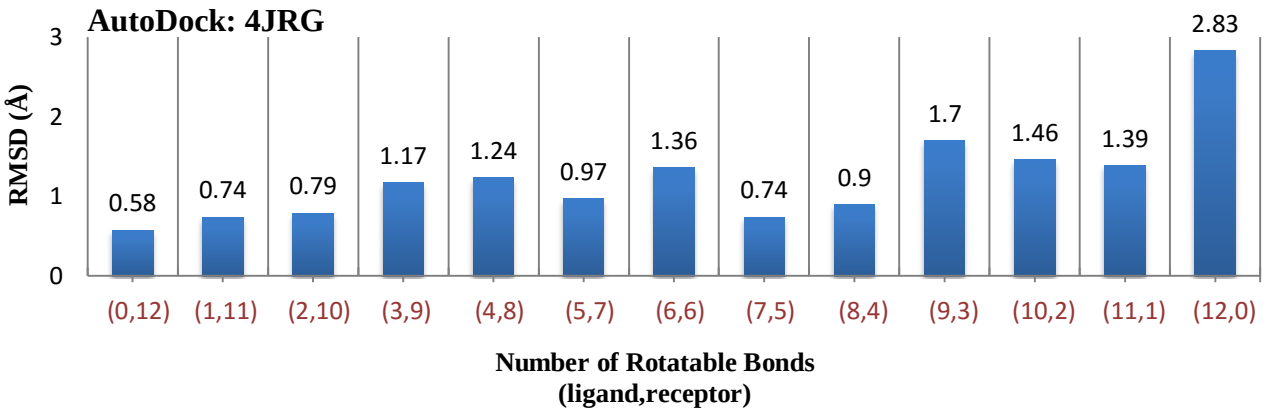

Fig. (4). The standard protocol (12,0) configuration shows an RMSD of 2.83 Å with the ligand having 12 rotatable bonds in contrast to a rigid ligand and all 12 rotatable bonds transferred to the MDM2 protein represented by configuration (0,12) with an RMSD of .58 Å.

from these structures are provided as supplemental materials (S2).

3.1 Implications for HTS

A notable result from this study is the large RMSD values for those configurations representing a completely flexible ligand with limited protein binding site flexibility. For 3LBK, this is docking run (5,7). Although this would seem the ideal distribution of flexibility as it incorporates the inherent flexibility of the ligand and binding protein residues, the RMSD value is 3.81 Å, far above the rigid ligand docking of .51 Å. The same is true of 4JRG, with the (10,2) configuration showing an RMSD of 1.46Å compared to .58Å. This is important because the (5,7) and (10,2) configuration of 3LBK and 4JRG respectively would be applicable to HTS as it does not require ligand modification or a change in protein flexibility once the screening starts, and would simulate in part, both protein and ligand binding dynamics more accurately than the standard protocol. This study finds a fully flexible ligand in combination with selective protein binding site flexibility fails to optimize the binding mode predictions. Docking results for structures 4IPF and 4ZYI, also show higher RMSD values for this distribution, with values of 1.64 Å and 3.75 Å respectively. An explanation for the disparity could be the consequence of re-docking a rigid ligand, and the built-in bias of the small molecule to assume the same binding mode as the experimentally known structure. However, this same bias is present using a rigid protein protocol, as the protein's binding site residues are essentially frozen in the optimal binding mode as is the rigid ligand. When the ligand and protein both have flexibility, this bias is partially removed and predicting binding modes close to the experimental structure becomes more difficult. In fact, all structures excluding 4JRG, produced higher RMSD values compared to both the standard and rigid ligand protocols when a fully flexible ligand was in combination with selected binding site flexibility. A more exhaustive examination of possible rotatable bond combinations simulating ligand and protein flexibility may, for any particular complex, improve the ligand binding mode prediction and improve RMSD values. The results from this study only indicates applying a rigid ligand protocol will produce lower RMSD values compared to the standard protocol for MDM2. This is of some importance considering the focus of medicinal interest on MDM2 and considering the amount of MDM2 targeted inhibitors developed as cancer therapeutics. For HTS, an exhaustive examination including all possible flexibility configurations is not a practical strategy as thousands of drug candidates are screened and evaluated containing varying chemical and physical properties, including flexibility.

3.1 Vina Results

The scoring function and search method employed by Vina has improved speed and binding mode predictions, and is probably better suited for HTS

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then AutoDock. However, AutoDock does hold some advantages over Vina in terms of modifying docking parameters and providing more detailed results. Vina's output of docking results are not nearly as extensive as AutoDock. For example, Vina supplies the top ten binding mode models in terms of binding free energy, but does not associate a RMSD value to the top ranked binding free energy. Consequently, Vina results are not charted for this study in terms of RMSD as with AutoDock. Confirmation of a successful docking using Vina and valid comparison to AutoDock must be accomplished using renderings of the binding mode in direct contrast to the experimentally determined structure. Comparing binding free energies is not applicable because AutoDock and Vina use different methods to determine binding free energy with both using many assumptions and estimates. This becomes especially problematic when adding flexibility as the loss of entropy is merely a weighted constant and not calculated.

An important advantage when using Vina along with a rigid ligand protocol for MDM2 is that it mitigates concerns of additional computational

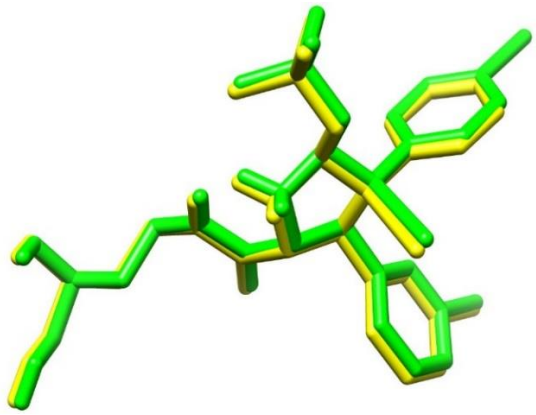

Fig. (5). Vina's prediction of rigid ligand docking of 4JRG (0,12) shown in green. This binding mode prediction is slightly closer to the experimentally known pose found by AutoDock in figure 6.

time and costs, while allowing for a flexible protein and more accurate binding prediction. Vina's multithreaded computer architecture can drastically reduce computational time while providing accurate results when docking ligands, as in this study, with 12 rotatable bonds. Vina's binding prediction for structure 4JRG mirrors AutoDock's best result of .58 Å (figures 5 and 6). However, Vina's calculation completed in less than 2 minutes, while the AutoDock calculation lasted 15 hours. The increased speed is not surprising, as this has been shown previously, but while transferring all flexibility to the protein, Vina, as with AutoDock, also produced a noticeable improvement of binding mode predictions as compared to the standard protocol. Applying a rigid ligand protocol when using Vina for HTS of MDM2 binding small molecules affords the consideration of larger ligands, while providing sufficient accuracy without increased computational costs.

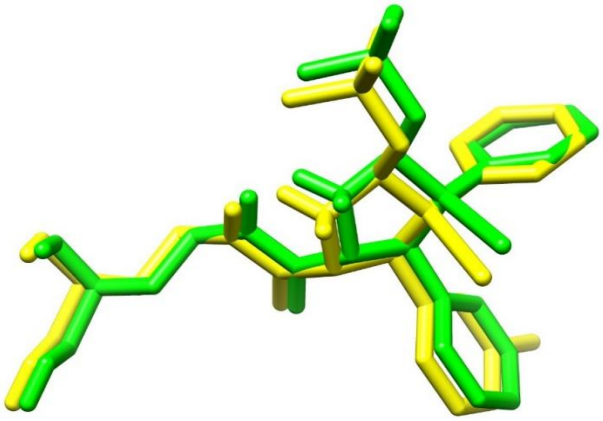

Fig. (6). AutoDock's prediction of rigid ligand docking of 4JRG (0,12) shown in green. This docked pose is .58 Å from the experimentally determined binding site (yellow).

3.2 Molecular Dynamics Analysis

The improved determination of binding geometries using a rigid ligand protocol is probably best explained by the physical and chemical structure of the P53 binding domain of MDM2. The binding domain pocket is flanked by residues not embedded within the protein. These residues can fluctuate during the binding process, allowing a rigid ligand to enter while the protein conforms according to interactions determined by the small molecules chemical structure (figure 7). A Classical MD simulation of the MDM2 protein using the crystallized structure 4IPF was performed by NAMD. A minimization and equilibration simulation allows for the determination of residue mobility before interaction with the ligand from average

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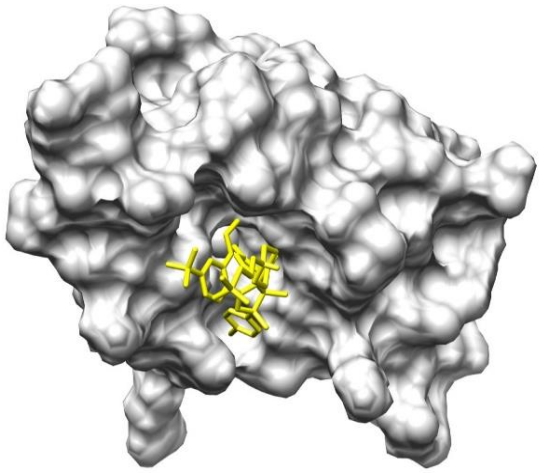

Fig. (7). MDM2 protein in complex with small molecule inhibitor. Human MDM2 is a 491- amino acid long phosphoprotein, whose p53 binding domain is found in a well-defined hydrophobic surface pocket encompassing a relatively small area of the protein. The binding pocket measures only 18 Å along the long edge; the size of a typical small molecule [8]. The MDM2 cleft is formed by amino acids 26–108, and consists of two structurally similar portions that fold up into a deep groove lined by 14 hydrophobic and aromatic residues [3].

values during equilibration. This data provides insight into the protein residue dynamics the ligand encounters as it enters the binding site. The protein was prepared and necessary files created using visual molecular dynamics (VMD), the graphical user interface designed to work with NAMD for the preparation, evaluation and visualization of MD simulations. The simulation lasted .5 ns to ensure equilibration with a 1 fs time step at a constant temperature of 310K in an explicit solvent. Results show the residues flexible for docking run (0,12) fluctuate between 1.4 and 3.2 Å, with residues HIS 69, HIS 92 and TYR 96 all moving an average of 3 Å (figure 8). The RMSD values serve to quantify movement of the protein in equilibrium, affirming the importance of modeling residue flexibility during docking experiments.

Although the flexibility as modeled by AutoDock and Vina is limited to the rotation of bonds, with bond lengths and angles kept constant, the change observed from flexible binding site residues can be pronounced. The before and after snapshot of binding site residues (figure 9) indicates the movement necessary to produce a successful docking within

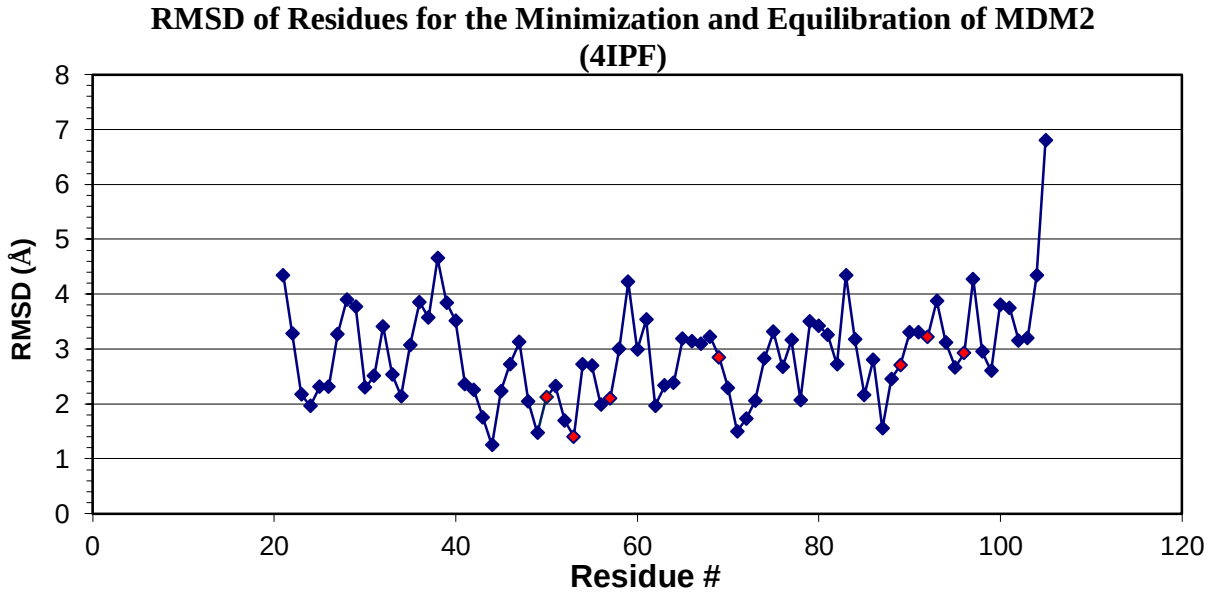

Fig. (8). The MDM2 protein as represented by crystal structure 4IPF was selected to evaluate fluctuation of residues providing quantitative data for a better understanding of the binding site dynamics. Red markers indicate flexible binding site residues.

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.49 Å of the known structure. This suggests standard protocol docking employing ligand flexibility may not be as essential to predicting an accurate binding mode as modeling the protein conformational changes accommodating a small molecule during the binding process. A short MD trajectory movie of 4IPF simulating protein movement is provided in the supplementary material (S3).

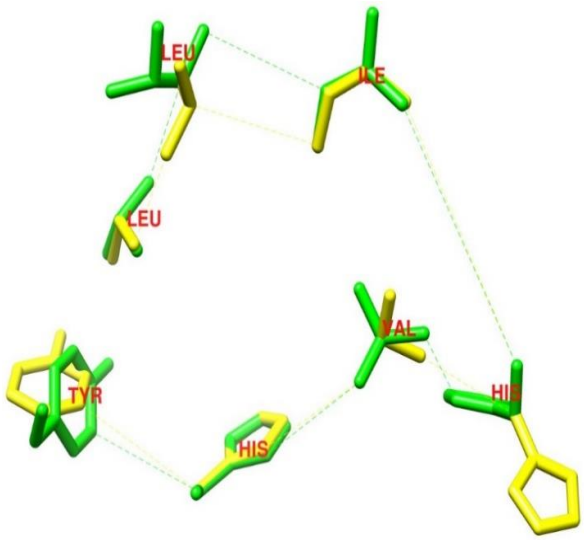

Fig. (9). Final positions of flexible binding site residues for 4IPF (0,12) shown in green, in contrast to pre-docking positions. Residues LEU, TYR, VAL and HIS have shifted considerably from the experimentally determined geometry. Due to the time independent nature of docking, we can only capture before and after states of the residues. The protein and ligand have been removed for clarity.

4. CONCLUSION

Improved binding mode predictions of small molecule inhibitors targeting the MDM2 protein was achieved using AutoDock and Vina employing a systematic distribution of 12 rotatable bonds between the ligand and protein. This study found a rigid ligand in combination with flexible binding site residues produced lower RMSD values from the known binding site when compared to standard rigid receptor docking. Further analysis of the flexible binding site residues found considerable movement illustrated by a MD simulation and examination of the predicted final positions and experimentally determined positions of the selected residues. Docking runs simulating a fully flexible ligand, in addition to rotatable bonds delegated to MDM2, failed to come as close as a rigid ligand configuration to the experimentally known structure. Adopting a rigid ligand/flexible protein docking protocol for future medicinal studies of MDM2 using Vina in particular, will enable accurate, fast predictions of MDM2 binding modes of small molecules with 12 and possibly more rotatable bonds. Future studies will determine if this protocol may be applied to other proteins or DNA targets of medicinal interest.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081).

SUPPLEMENTARY MATERIAL

S1. Docking input files

S2. Docking results for structures 4ipf and 4zyi

S3. Molecular dynamics 4ipf video

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