Biochemical and Biophysical Research Communications 370 (2008) 547–551

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Aromatic–aromatic interactions in the formation of the MDM2–p53 complex

L. Michel Espinoza-Fonseca [a,b,*], Jazmín García-Machorro [c]

[a] Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455, USA
[b] Departamento de Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. Carpio y Plan de Ayala, Mexico City 11340, Mexico
[c] Departamento de Biomedicina Molecular, Cinvestav-IPN, Mexico City 07360, Mexico

ARTICLE INFO

Article history:
Received 6 March 2008
Available online 18 March 2008

Keywords:
MDM2
p53
Molecular dynamics simulations
Aromatic interactions
Cancer
Apoptosis

ABSTRACT

Although the molecular interaction of MDM2 with the transactivation domain of p53 has been thoroughly studied, there is very limited information regarding the steps involved in the recognition mechanism between these proteins. On this basis, we performed four high-temperature molecular dynamics simulations in explicit solvent to gain insight into the interactions involved in the fist contact toward the formation of the complex. We found that the presence of specific intermolecular aromatic pairs at the interface of the complex, around the native-like state of MDM2, is consistent among independent molecular dynamics runs. This observation suggests that aromatic–aromatic interactions are closely related to the first contact between MDM2 and p53. Thus, we propose that aromatic–aromatic interactions are an important, and probably essential, requirement for the formation and stabilization of the MDM2–p53 complex.

© 2008 Elsevier Inc. All rights reserved.

The mouse double minute protein 2 (MDM2) plays a crucial role in the regulation of p53, a transcription factor that is pivotal to cellular responses due to stress in the cell [1]. MDM2 regulates p53 through (a) the inhibition of the transcriptional activity of p53 (by directly binding to a region within the N-terminus of p53) and (b) its degradation via the ubiquitin–proteasome pathway [2]. Hence, an overexpression of MDM2 is known to produce a loss of wild-type p53 activity in cancer cells, allowing tumor cells to escape from p53-induced apoptosis.

The resolution of the X-ray structure of the region of MDM2 that binds the transactivation domain of p53 (residues Glu17 to Asn29) revealed interesting features regarding the nature of the interaction between these two proteins [3]. The p53-binding region of MDM2 is constituted by two pairs of α-helices that form a hydrophobic cleft where p53 binds. The stabilization of the MDM2–p53 complex is mainly produced by the anchoring of two aromatic residues of p53 (Phe19 and Trp23) to this hydrophobic cleft. Further experimental [4,5] and computational [6,7] studies have confirmed the importance of a few residue–residue interactions in the thermodynamics of binding. Given the availability of the three-dimensional structure of the complex, it was suggested that perturbing the interaction between MDM2 and p53 by using small synthetic molecules could represent a potential alternative for cancer therapy [8]. Following up with this idea, and considering the molecular basis of the interaction between MDM2 and p53, anti-cancer drug design efforts became more tangible, culminating with the remarkable discovery of nutlins, a group of *cis*-imidazoline-derived molecules that were able to bind with high affinity to MDM2 [9].

Although a vast body of information is available regarding the stability of the MDM2–p53 complex, our understanding of the nature of recognition between the two proteins still remains very limited. On this basis, through high-temperature molecular dynamics (MD) simulations in explicit solvent, we directly showed for the first time—at least to our knowledge—the relevance of aromatic interactions in the recognition mechanism of p53 to MDM2. We found that the presence of specific intermolecular aromatic pairs at the interface of the complex, around the native-like state of MDM2, is consistent among independent MD runs. This observation suggests that aromatic–aromatic interactions are closely related to the first contact between MDM2 and p53.

Computational methods

Preparation of the MDM2–p53 complex. The three-dimensional structure of *Xenopus laevis* MDM2 bound to the 11-aminoacid segment of the transactivation domain of human p53 (PDB code: 1YCQ) was used for the MD simulations. The protonation states of ionizable side chains were adjusted to a pH of 7.0 by using PROPKA [10]. Hydrogen atoms were added to the structure with the aid of PSFGEN, with no optimization of the hydrogen bond network. The complex was further inserted in a TIP3P water box with a margin of at least 20 Å between the complex and the boundaries of the periodic box. Chlorine and sodium counter

* Corresponding author. Address: Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455, USA.
E-mail address: mef@ddt.biochem.umn.edu (L.M. Espinoza-Fonseca).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.03.053

ions were added to neutralize the charge of the system and to produce ionic strength of approximately 150 mM. The entire system was modeled with the CHARMM25 force field [11,12].

Molecular dynamics simulations of the complex. In order to prepare the system for the MD simulations, the system was subjected to an equilibration period. Prior to equilibration, the protein was first minimized by using 500 steps of conjugate gradient with a restraints on the protein heavy atoms, followed by 500 steps of conjugate gradient with no restraints. The system was further warmed up to a target temperature of 400 K for 80 ps under a NVT ensemble. Finally, a full equilibration of the system was achieved by applying harmonic restraints to the alpha carbons of the protein; such restraints were smoothly removed throughout the 100 ns of equilibration period. For equilibration purposes, the NVT ensemble was swapped by a NPT ensemble. A total of four independent 50-ns runs were obtained by randomly assigning initial velocities to the atoms in the systems. MD simulations were performed by using the program NAMD 2.6 [13].

Results and discussion

The main aim of using high-temperature MD simulations is to enhance the sampling of the conformational and configurational space of the complex near to its native-like state. Particular emphasis was given to the role of aromatic residues in the first contact between the two proteins. For that purpose, independent high-temperature MD simulations of the complex between the MDM2 from *X. laevis* and p53 from human were performed. Moreover, the use of a hybrid complex is of great importance because it can reveal highly conserved features intrinsic to the recognition mechanism of p53 by MDM2.

In order to evaluate the stability of MDM2 (the target protein) in the simulations, plots of the root-mean square deviation (RMSD) of the alpha carbon trace were obtained (Fig. 1). It was observed that most of the conformations explored in the four simulations satisfied a RMSD $\leqslant$ 5 Å. By comparing the number of native contacts between a MD simulation of the complex at 310 K [14] and the four simulations at 400 K, it was observed that at least 80% of the native contacts may be found in structures with a RMSD difference of 5 Å. On this basis, the RMSD plots indicated that the conformational changes explored in our simulations corresponded to the sub-domain motions of MDM2 around its native-like state. If we ignore the N- and C-termini of MDM2, the major contribution to the RMSD in the four simulations comes from the large motions of the loop/β-sheet domain that connects helices II and III. A visual inspection of the secondary structure of MDM2 throughout the four independent simulations revealed that there was no dramatic change in the fold of helices I–IV, confirming that the temperature-induced sampling acceleration did not significantly affect the native-like folding of the binding cleft of MDM2.

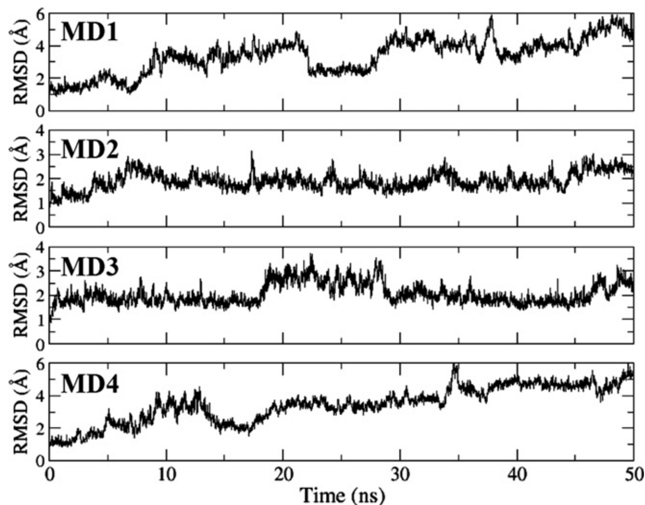

Fig. 1. Root-mean square deviations of the alpha carbon trace of MDM2 for each independent MD simulation.

The region of p53 that binds to MDM2 experienced large fluctuations in the RMSD throughout the simulations. However, these changes were directly associated to the translational motions of the peptide. Analysis of the secondary structure of p53 showed that the stability of the α-helix constituted by residues F19-K24 remains stable during most of the simulation length in each independent MD simulation (α-helical content $\geqslant$80% of the total simulation time). This observation is in good agreement with experimental and computational studies, which have reported the presence of a stable helix within the intrinsically unstructured transactivation domain of p53 [15,16].

To further test the native-like nature of MDM2 in our simulations, the root-mean square fluctuations (RMSF) of the alpha carbons were computed. The resulting plots (Fig. 2) showed that MDM2 in each simulation retained structural features observed in previous simulations of the MDM2–p53 complex at 310 K [14]. For example, the loop/β-sheet region formed by residues Leu62-His72 displayed higher flexibility compared to the rest of the protein. In general, the helices I–IV of MDM2 and the loops that connect them remained relatively rigid (RMSF $\leqslant$ 2 Å) in the simulations. Only the segment Leu80-Gln85 in one of the simulations (MD4, Fig. 2) appeared to have larger fluctuations, which corresponded to the partial unfolding of this region.

By using high-temperature MD simulations, we expected to observe at least a partial unbinding of p53 from MDM2. Hence, a given target temperature in the nanosecond timescale must yield a relatively fast unbinding, yet allowing local interactions at the protein–protein interface to be explored. Moreover, such unbinding should not be accompanied by a significant loss of the native interactions of individual partners. Considering that most of the conformational space sampled in the four independent simulations corresponded to native-like states of MDM2, we proceeded to detect the possible pair–pair aromatic interactions between p53 and MDM2.

Although various intermolecular aromatic pairs were found in the course of the simulations, only a few of them seemed to play a relevant role in binding p53 to MDM2. These pairs were formed by Phe19 from p53 and Tyr51 from MDM2; Phe19 from p53 and Tyr63 from MDM2; and Trp23 from p53 and Tyr51 from MDM2. Other aromatic pairs were observed transiently, but they were not included in the analysis because their participation was not significant in the binding mechanism. Thus, our analysis was mainly focused on the three aromatic pairs described above. The percentage of time spent in *specific* (i.e., strictly stacked or T-shaped π–π interactions) and *non-specific* (i.e., a mixture of weak

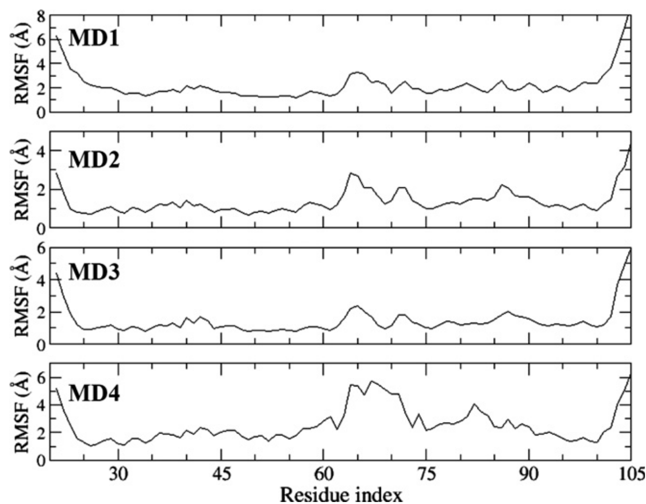

Fig. 2. Root-mean square fluctuations of individual residues of MDM2.

π–π and hydrophobic interactions) interactions between aromatic pairs was calculated (Fig. 3). *Specific* interactions between aromatic pairs were defined by those interactions that satisfy a centroid–centroid distance $r \leqslant 5.5$ Å and interplanar angle of 0–90°; these values were suggested from calculated equilibrium geometries for pairs of aromatic aminoacids [17]. For *non-specific* aromatic interactions, only the centroid–centroid cutoff distance interval 5.6 Å $\leqslant r \leqslant$ 7.5 Å was used.

The analysis of both specific and *non-specific* interactions between relevant aromatic pairs showed a similar trend in the four simulations; for example, the percentage of time spent in *specific* and *non-specific* aromatic interactions for the pair Phe19/Tyr63 is very similar in simulations MD1 and MD3 (∼50% of the total time); likewise, in simulations MD1 and MD4, the pair Trp23/Tyr51 spent an average of 65% of the total time in both *specific* and *non-specific* interactions (Fig. 3). If we consider the total contribution of *specific* and *non-specific* interactions of aromatic residues, we can infer that aromatic pairs significantly contribute to the binding of p53 to MDM2. However, the information extracted from the total contribution of *specific* and *non-specific* aromatic interactions may be rather limited and might not reflect the actual participation of π–π interactions. Therefore, the contribution of *specific* aromatic interactions was calculated (Fig. 3, red bars). Interestingly, the contribution of π–π interactions per pair to the binding of p53 to MDM2 seemed to follow a very similar pattern in the simulations, as it can be observed in Fig. 3. Hence, the pair Trp23/Tyr51 had the largest percentage of time spent in π–π interactions in all simulations, going from ∼16% in simulation MD2 to ∼33% in simulation MD3. The pair constituted by Phe19/Tyr63 is the second most dominant type of π–π interaction in the complex, ranging from ∼7% in MD2 to ∼16% in MD1. It is important to note that a stacked-type π–π interaction between Phe19 and Tyr63 has been observed in the crystal structure of the complex, indicating that Tyr63 of MDM2 not only plays a role in the molecular recognition mechanism of p53, but also in the stability of the protein–protein complex. Finally, the formation of the Phe19/Tyr51 pair was also observed in our simulations. Even though the percentage of time spent in π–π interactions was found to be low (from ∼1.5% in MD3 to ∼7% of the time in MD4), the existence of this transient aromatic–aromatic complex may have a very important functional role in the first contact between MDM2 and p53, as it will be discussed in the following paragraphs.

Considering the results presented above, two questions arise from this study: (a) are aromatic–aromatic interactions relevant in the interaction between MDM2 and p53? If so, (b) how do these interactions participate in the binding mechanism of p53 to MDM2? To answer these questions, we have to take into consideration that π–π interactions have an utmost contribution in drug–receptor interactions, the structural stability of nucleic acids and protein folding [18]. Although the role of aromatic residues in the stability of protein–protein complexes has not been thoroughly studied, it is becoming more accepted that such interactions actively participate in the formation and stability of protein–protein complexes [19]. Despite the fact that the energetic contribution of aromatic–aromatic interactions may be small compared to the total enthalpy of binding, aromatic residues can serve as scaffolds that guide binding partners toward a low energy, stable complex. Such might be the case of the binding of p53 to MDM2. Hence, the answer to the first question is affirmative. We propose that the participation of aromatic–aromatic interactions in the formation of the complex is focused in the first contact between p53 and MDM2. How do aromatic interactions participate in this first contact? Both NMR experiments and MD simulations have shown that the p53-binding cleft of MDM2 is "closed" in the free state of the protein [14,20]. Moreover, the structure of the MDM2–p53 complex shows that residue Trp23 from p53 is buried in the cleft of MDM2. This suggests that the formation of the complex may involve at least two intermediate states, where the formation of first contacts between the two proteins is followed by the opening of the cleft of MDM2 and the formation of the stable complex. To better illustrate this hypothesis, and assuming that the unbinding pathway is the reverse of binding, we propose a binding mechanism that heavily relies on aromatic interactions (Fig. 4).

The first step of the recognition mechanism can be described as the formation of an aromatic cluster between Phe19 and Trp23 from p53 and the solvent-exposed Tyr51 from MDM2 (Fig. 4A).

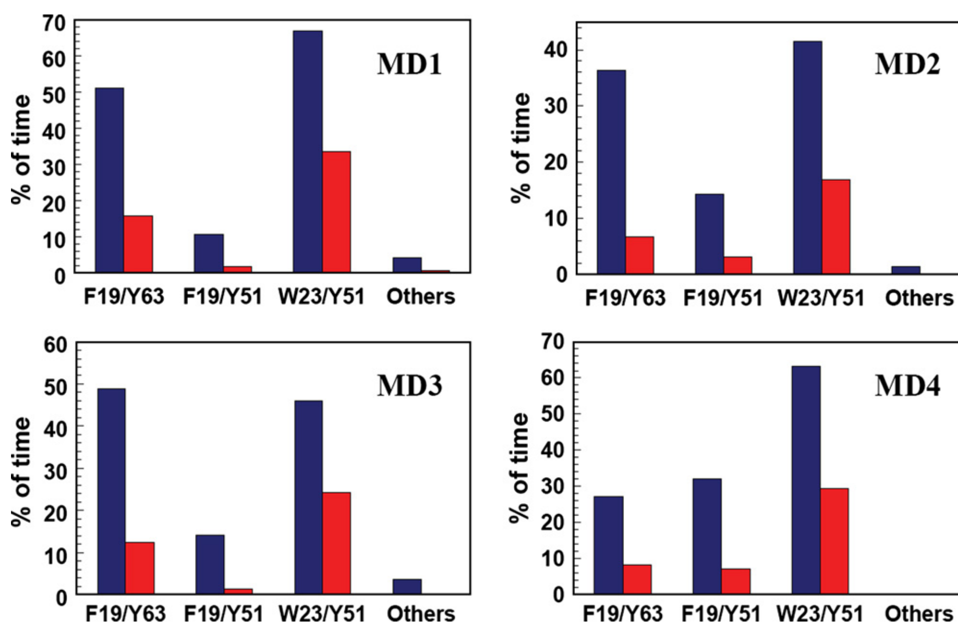

Fig. 3. Percentage of time spent in *specific* and *non-specific* aromatic interactions (blue) and purely *specific* aromatic interactions (red). Only pairs of relevant aromatic residues are shown. Definitions of *specific* and *non-specific* aromatic interactions are given in the main text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

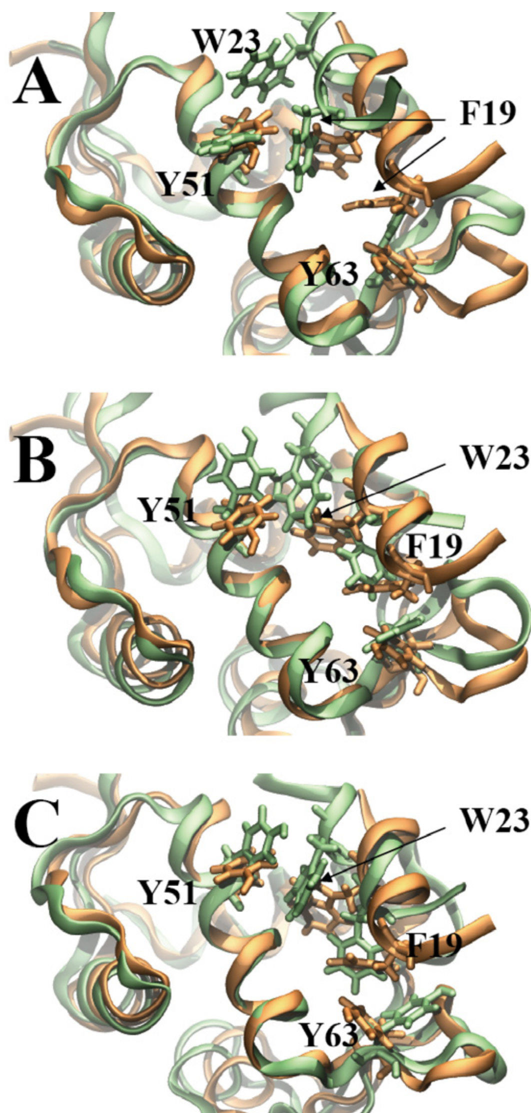

Fig. 4. Proposed mechanism of binding between MDM2 and p53. Selected snapshots from our trajectories (lime) were superimposed to the average structure of the complex taken from a simulation at 310 K (orange) [14]. Both MDM2 and p53 are rendered as ribbons, and selected aromatic residues are shown as sticks. (A), (B) and (C) correspond to different steps of the binding mechanism. A detailed description of each step is given in the main text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We hypothesize that the formation of this complex is transient, considering the low percentage of time spent in the interaction Phe19/Tyr51 (Fig. 3). The formation of such cluster might be of remarkable importance in the first recognition by p53, considering that an aromatic residue is also found in this region of human MDM2 (Phe55). A second step involves the diffusion of p53 toward the cleft of MDM2; this step may be facilitated by *specific* aromatic interactions between Trp23 from p53 and Tyr51 from MDM2 as well as by *non-specific* aromatic interactions between Phe19 from p53 and Tyr63 from MDM2 (Fig. 4B). During this step, the opening of the binding cleft of MDM2 could be induced by means of steric repulsions. In a final step, *non-specific* aromatic interactions between Trp23 and Tyr51 may assist the accommodation of the former in the cleft of MDM2; furthermore, *specific* interactions taking place between Phe19 and Tyr63 might favor the steering of the peptide in order to produce a stable complex. This last step is depicted in Fig. 4C. It is worth mentioning that, despite $-CH_3{\cdots}\pi$ interactions were not discussed in this study, they were observed (at least transiently) in our trajectories, suggesting a possible participation in the binding mechanism.

Conclusion

Overall, the results presented in this study explain, in part, the mechanism of first recognition between MDM2 and p53 via aromatic–aromatic interactions. The results also suggest that the presence of solvent-exposed aromatic residues in both p53 and MDM2 has a relevant functional role in the formation of the MDM2–p53 complex. Furthermore, the use of a hybrid complex (i.e., two proteins from different species) strongly suggests that the recognition mechanism involving both *specific* and *non-specific* aromatic interactions is quite conserved along the phylogenetic tree. Considering these observations, we hypothesize that aromatic–aromatic interactions are an important, and probably essential, requirement for the formation and stabilization of the MDM2–p53 complex. This hypothesis awaits confirmation by further experimental studies. For example, binding kinetics of the MDM2 holding the mutation Tyr51K → Ala51 (from *X. laevis*) or Phe55 → Ala55 (from human) may be performed. Additional studies will also unveil the contribution of $-CH_3{\cdots}\pi$ interactions to the binding of p53 to MDM2.

This study may also be helpful for the design of anti-cancer drugs. A more specific, high-affinity targeting of MDM2 can be achieved by using aromatic moieties as anchors. This approach was recently explored by Fasan et al. The authors reported the X-ray structure of the complex between human MDM2 and a synthetic peptide, cyclo-(L-Pro-Phe-Glu-6-chloroTrp-Leu-Asp-Trp-Glu-Phe-D-Pro) [21]. Interestingly, the formation of an aromatic cluster between Phe55 from human MDM2 and two aromatic residues from the cyclopeptide was observed; the affinity of this cyclopeptide was found to be 1000 times higher compared to its lead β-hairpin mimetic. Based on structural evidence, the authors suggested that this improvement in the affinity arose from the formation of such aromatic cluster.

Finally, this study constitutes a good example of using high-temperature MD simulations to gain insight into the specific features of recognition in protein–protein complexes in atomic detail. Although this approach is not flawless, it still provides a relatively unbiased description of the possible role of specific residues (such as aromatic ones) in the formation of protein–protein complexes.

Acknowledgments

L.M.E.F. was supported in part by grants from the Minnesota Supercomputing Institute and the Barcelona Supercomputing Center (QCM-2007-2-0003 and QCM-2007-3-0005). The authors acknowledge the computer resources, technical expertise and assistance provided by the Barcelona Supercomputing Center. J.G.M. is a recipient of a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACYT).

References

[1] J. Momand, H.H. Wu, G. Dasgupta, MDM2-master regulator of the p53 tumor suppressor protein, Gene 242 (2000) 15–29.
[2] A.J. Levine, P53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.
[3] P.H. Kussie, S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A.J. Levine, N.P. Pavletich, Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain, Science 274 (1996) 948–953.
[4] Z. Lai, K.R. Auger, C.M. Manubay, R.A. Copeland, Thermodynamics of p53 binding to hdm2(1–126): effects of phosphorylation and p53 peptide length, Arch. Biochem. Biophys. 381 (2000) 278–284.
[5] O. Schon, A. Friedler, S. Freund, A.R. Fersht, Molecular mechanism of the interaction between MDM2 and p53, J. Mol. Biol. 323 (2002) 491–501.

[6] I. Massova, P.A. Kollman, Computational alanine scanning to probe protein–protein interactions: a novel approach to evaluate binding free energies, J. Am. Chem. Soc. 121 (1999) 8133–8143.
[7] H. Zhong, H.A. Carlson, Computational studies and peptidomimetic design for the human p53–MDM2 complex, Proteins 58 (2005) 222–234.
[8] P. Chene, Inhibiting the p53–MDM2 interaction: an important target for cancer therapy, Nat. Rev. Cancer 3 (2003) 102–109.
[9] L.T. Vassilev, B.T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E.A. Liu, In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, Science 303 (2004) 844–848.
[10] H. Li, A.D. Robertson, J.H. Jensen, Very fast empirical prediction and interpretation of protein pK_a values, Proteins 61 (2005) 704–721.
[11] A.D. MacKerell Jr., D. Bashford, M. Bellott, R.L. Dunbrack Jr., J.D. Evanseck, M.J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, D. Joseph-McCarthy, L. Kuchnir, K. Kuczera, F.T.K. Lau, C. Mattos, S. Michnick, T. Ngo, D.T. Nguyen, B. Prodhom, W.E. Reiher III, B. Roux, M. Schlenkrich, J.C. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorkiewicz-Kuczera, D. Yin, M. Karplus, All-atom empirical potential for molecular modeling and dynamics studies of proteins, J. Phys. Chem. B 102 (1998) 3586–3616.
[12] A.D. MacKerell Jr., M. Feig, C.L. Brooks III, Improved treatment of the protein backbone in empirical force fields, J. Am. Chem. Soc. 126 (2004) 698–699.
[13] J.C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R.D. Skeel, L. Kale, K. Schulten, Scalable molecular dynamics with NAMD, J. Comput. Chem. 26 (2005) 1781–1802.
[14] L.M. Espinoza-Fonseca, J.G. Trujillo-Ferrara, Conformational changes of the p53-binding cleft of MDM2 revealed by molecular dynamics simulations, Biopolymers 83 (2006) 365–373.
[15] H. Lee, K.H. Mok, R. Muhandiram, K.H. Park, J.E. Suk, D.H. Kim, J. Chang, Y.C. Sung, K.Y. Choi, K.H. Han, Local structural elements in the mostly unstructured transcriptional activation domain of human p53, J. Biol. Chem. 275 (2000) 29426–29432.
[16] S. Kar, K. Sakaguchi, Y. Shimohigashi, S. Samaddar, R. Banerjee, G. Basu, V. Swaminathan, T.K. Kundu, S. Roy, Effect of phosphorylation on the structure and fold of transactivation domain of p53, J. Biol. Chem. 277 (2002) 15579–15585.
[17] F.L. Gervasio, R. Chelli, P. Procacci, V. Schettino, The nature of intermolecular interactions between aromatic amino acid residues, Proteins 48 (2002) 117–125.
[18] A.K. Tewari, R. Dubey, Emerging trends in molecular recognition: utility of weak aromatic interactions, Bioorg. Med. Chem. 16 (2008) 126–143.
[19] C. Yan, F. Wu, R.L. Jernigan, D. Dobbs, V. Honavar, Characterization of protein–protein interfaces, Protein J. 27 (2008) 59–70.
[20] S. Uhrinova, D. Uhrin, H. Powers, K. Watt, D. Zheleva, P. Fischer, C. McInnes, P.N. Barlow, Structure of free MDM2 N-terminal domain reveals conformational adjustments that accompany p53-binding, J. Mol. Biol. 350 (2005) 587–598.
[21] R. Fasan, R.L. Dias, K. Moehle, O. Zerbe, D. Obrecht, P.R. Mittl, M.G. Grütter, J.A. Robinson, Structure–activity studies in a family of beta-hairpin protein epitope mimetic inhibitors of the p53–HDM2 protein–protein interaction, Chembiochem 7 (2006) 515–526.