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RESEARCH ARTICLE

Structural effects and competition mechanisms targeting the interactions between p53 and MDM2 for cancer therapy

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Approximately half of all human cancers show normal TP53 gene expression but aberrant overexpression of MDM2 and/or MDMX. This fact suggests a promising cancer therapeutic strategy in targeting the interactions between p53 and MDM2/MDMX. To help realize the goal of developing effective inhibitors to disrupt the p53–MDM2/MDMX interaction, we systematically investigated the structural and interaction characteristics of p53 with inhibitors of its interactions with MDM2 and MDMX from an atomistic perspective using stochastic molecular dynamics simulations. We found that some specific α helices in the structures of MDM2 and MDMX play key roles in their binding to inhibitors, and that the hydrogen bond formed by the Trp23 residue of p53 with its counterpart in MDM2 or MDMX determines the dynamic competition processes of the disruption of the MDM2–p53 interaction and replacement of p53 from the MDM2–p53 complex *in vivo*. The results reported in this paper are expected to provide basic information for designing functional inhibitors and realizing new strategies of cancer gene therapy.

Keywords p53, MDMX, MDM2, molecular dynamics simulation, inhibitors, cancer therapy

PACS numbers 89.75.-k, 82.39.Rt, 87.15.km, 87.15.ap

1 Introduction

p53, a tumor suppressor protein, is considered to be the guardian of the genome and plays a crucial role in regulation of the cell cycle, apoptosis, DNA repair, and angiogenesis [1]. Increases in the p53 level cause cell cycle arrest or apoptosis in response to the signal of DNA damage. The activation of p53 can further activate another protein, MDM2, which negatively regulates the level of p53. In normal cells, the expression levels of both MDM2 and p53 are low, which provides a growth advantage. However, approximately half of all human cancers show normal TP53 gene expression but aberrant overexpression of MDM2 and/or MDMX [2], which inhibit the activation of p53. This fact suggests a new potent strategy for cancer therapy, i.e., restoration of p53 activity by competitive inhibitors that can occupy the p53-binding site of MDM2 and thus inhibit the interaction between p53 and MDM2. Once freed from MDM2, p53 rapidly accumulates in the nuclei of cancer cells, activates p53 target genes and the p53 pathway, thereby resulting in cell-cycle arrest and apoptosis [3–5].

In history, it has been difficult to develop small-molecule inhibitors of non-enzyme protein–protein interactions [3]. Protein–protein interactions usually involve large and flat surfaces that are difficult to break by low-molecular-weight compounds [6, 7]. However, the crystal structure of MDM2 bound to a peptide from the transactivation domain of p53 revealed that MDM2 possesses a relatively deep hydrophobic pocket that is filled primarily by three side chains from the helical region of the peptide [4]. The existence of such a well-defined pocket on the MDM2 molecule suggested that compounds with low molecular weights could be identified and developed

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to block the interaction between MDM2 and p53.

The design of such inhibitors should follow the principle to closely model the MDM2-binding site of p53 with respect to the characteristic features of the interaction [5]. p53 and MDM2 mainly associate through hydrophobic interactions and hydrogen bonds. Three residues of p53, Phe19, Trp23, and Leu26, have been found to be essential for the binding between p53 and MDM2, and are inserted into a deep hydrophobic pocket on the surface of the MDM2 molecule. These features of interactions between p53 and MDM2 should be maintained in the design of an efficient inhibitor. The interface between p53 and MDM2 is small, which permits the design of relatively small inhibitors with high oral bioavailability [8].

Recently, rational designing with molecular docking and high-throughput virtual screening approaches have led to the generation of a diversified set of small molecules and peptides that can restore the activity of p53 [5, 9, 10]. Members of the Nutlin family are among these inhibitors, which are non-polypeptide inhibitors, including Nutlin1, Nutlin2, Nutlin3, and RG7112 [11]. Nutlin3 is one of the most promising inhibitors, which is currently under clinical investigation. Nutlin3 has been shown to tightly combine with MDM2 and efficiently inhibit the p53–MDM2 interaction [12].

Moreover, MDMX (Mdm4 in mice) is another negative regulator of p53, which controls the stability and activity of p53 via a different mechanism from MDM2 [13]. MDMX is highly homologous to MDM2, with the key difference being that it does not possess any ubiquitin ligase activity and does not cause p53 degradation, although it binds to the N-terminus of p53 and suppresses p53 transcriptional activities [14]. Despite the high degree of homology between MDM2 and MDMX, especially in their N-terminal p53-binding domains, existing inhibitors of the MDM2–p53 interaction show weak activity to inhibit the interactions between MDMX and p53, thus highly reducing the effect of these inhibitors in cancer therapy. Hence, it is important to develop dual small-molecule inhibitors of p53–MDM2 and p53–MDMX interactions to achieve the complete reactivation of p53 [15].

To this end, the structural characteristics and properties of the inhibitor–MDM2 and inhibitor–MDMX interactions should be systematically investigated. Considering the fact that the currently used docking scoring functions are not expected to provide consistently accurate predictions of the protein–ligand binding free energies for all protein–ligand binding systems, and the effects of protein dynamics on microscopic binding are difficult to account for using the simple docking process [16], we instead employed molecular dynamics (MD) simulations, which can more reasonably account for the solvation effects and the dynamics of the protein–ligand binding. Use of MD simulations allowed us to obtain a dynamically stable protein–ligand binding mode associated with a stable MD trajectory.

In this study, we set up four models (p53–MDM2, p53–MDMX, Nutlin3–MDM2, and Nutlin3–MDMX) to investigate the interactions between p53 and MDM2 or MDMX and between Nutlin3 and MDM2 or MDMX using MD simulations through comparison of interactions between MDM2/MDMX and p53 or the inhibitor. We found that the binding patterns of p53–MDM2 and p53–MDMX are essentially the same, despite the differences in their sequences and structures. Moreover, in contrast to the tight binding of Nutlin-3 to MDM2, Nutlin-3 cannot bind to MDMX because of three factors arising from the sequence differences between MDM2 and MDMX.

2 Methods

For development of the model, complexes of p53–MDM2 (PDB ID: 1YCR [4]), p53–MDMX (PDB ID: 3DAB [17]), and Nutlin3–MDM2 (PDB ID: 4HG7 [18]) were extracted from the corresponding PDB files and solvated by explicit water molecules, respectively. The radius of the water sphere was 10 Å larger than that of the proteins. Because there is still no crystal structure of the Nutlin3–MDMX complex available, we replaced the MDM2 molecule of 4HG7 by the MDMX molecule to construct the Nutlin3–MDMX complex. This complex was also buried by explicit water molecules.

The ionic concentration used was 150 mM. TIP3P [19] was used to model water molecules. The protein–water complexes were subjected to 30 000 steps of energy minimization, and then 50-nanosecond (ns) MD simulations were performed. In our MD simulations, the α-carbons of Ile500, Asp67, and Leu109 in MDMX and those of Glu25 and Val109 in MDM2 were fixed. The models were developed using VMD (version 1.9) [20], and MD simulations were performed with NAMD code (version 2.9) [21] with the force file CHARMM [22] at a constant temperature of 310 K. The non-bonded Coulomb and van der Waals interactions were calculated with a cutoff using a switching function starting at a distance of 13 Å, reaching zero at 15 Å. The integration time step was 2 femtoseconds (fs).

3 Results and discussion

3.1 Comparison of the sequences and structures between MDMX and MDM2

The functions of a biomolecule are mainly determined by its structure. Therefore, as a starting point, we inves-

tigated the sequence and structure differences between MDMX and MDM2. The fact that existing MDM2 inhibitors have weaker binding affinity for MDMX protein [23–25] may indicate that some structural differences between the p53-binding domains of MDM2 and MDMX play a crucial role in the binding properties of inhibitors to MDM2 and MDMX [12, 15].

To investigate such structural differences, we compared the amino acid sequences and the tertiary structures of the p53-binding domains between MDM2 and MDMX, as shown in Fig. 1. The degree of sequence identity between the N-termini of MDM2 and MDMX was found to be 53.9%, and the differences between them can be classified into three types according to changes in the polarity (∼36.6%), hydrophobicity (∼19.5%), and geometric structures of the side chains (∼39%). There are four main regions in their backbones that show relatively large differences (C_αs' distances larger than 0.7 Å) between the proteins, residues Ser40 to Met50, Thr63 to Gln71, Ser78 to Phe86, and Lys94 to Arg105 in MDM2 [Fig. 1(b) and Fig. 1(c)], whereas the superposition of tertiary structures of the corresponding region between MDM2 and MDMX showed high similarity. The segments of Ser40 to Met50 and Thr63 to Gln71 are loops in the secondary structures, which show large flexibility in a water environment [26]. Therefore, the structural difference between MDM2 and MDMX is mainly caused by sequence differences on two helices, H3 (MDM2; residues 78-84) and H4 (MDM2; residues 94-106), where H4 is the key element of the p53-binding sites of both MDM2 and MDMX.

Note that there is an obvious difference in the α helix H1s of MDM2 and MDMX. From the crystal structures of p53–MDM2, H1 of MDM2 covers the p53-binding site

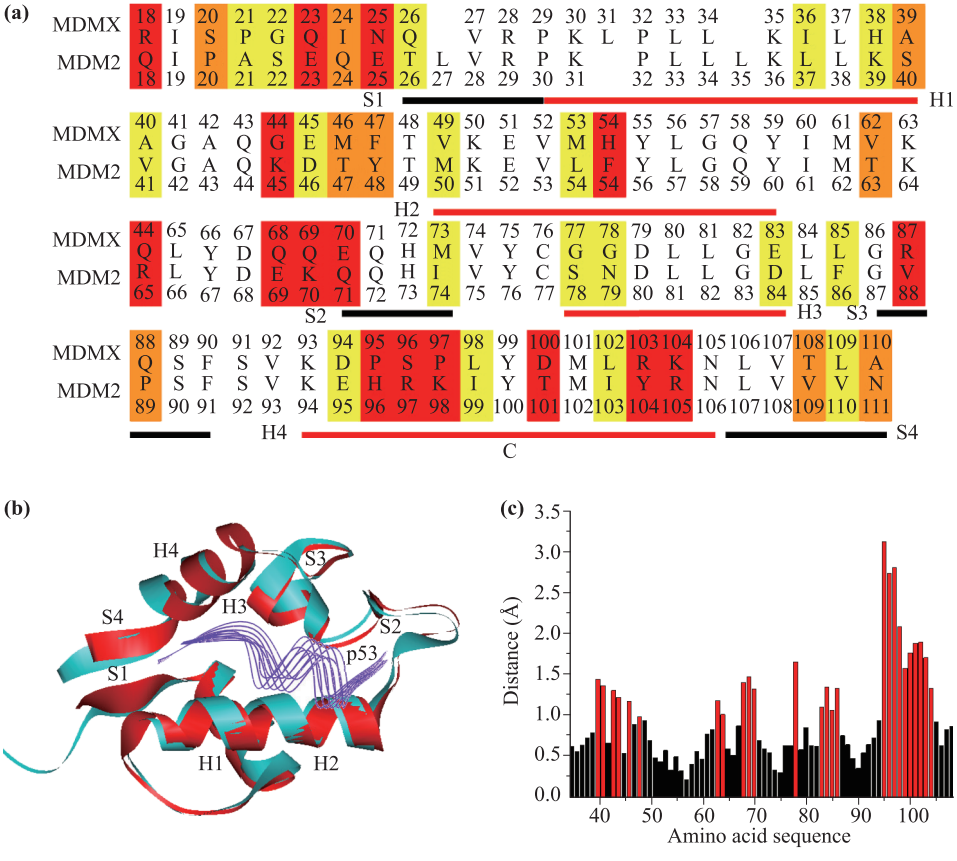

Fig. 1 Comparison of the sequences and structures of the p53-binding sites of MDM2 and MDMX. **(a)** Amino acid sequence alignment of the p53-binding sites of MDM2 and MDMX. The different types of amino acid changes are highlighted in different colors representing changes of polarity (red), hydrophobicity (brown), and geometric structures (yellow). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. **(b)** Superposition of the crystal structures of the N-termini of MDM2 (red) and MDMX (green). p53 is depicted with purple lines. Large structural differences are apparent at the two loop regions and two α helices (H3 and H4). **(c)** Each pair of C_α distances of the structure shown in (b). The red bars show the corresponding C_α pairs with distances longer than 1 Å. Because of the uncertainty of the H1 structure of MDMX, comparison of the distance between the two H1s is not shown.

of MDM2, which indicates the potential function of H1 in the binding between the proteins. However, H1 of MDMX is absent in the crystal structures and we could not identify its exact position. We will discuss the potential roles of helix H1 further in Section 3.3.

3.2 Binding patterns of p53 to MDM2 and MDMX

It is also important to consider that the structures and functions of a biomolecule are bridged by dynamical process occurring from the micro to macro scales. To clarify why the above-mentioned structural difference results in different binding characteristics between p53–MDM2 and p53–MDMX and between inhibitor–MDM2 and inhibitor–MDMX interactions, we first compared the binding pattern of p53 to MDM2 and MDMX with MD simulation. It is known that both MDM2 and MDMX proteins can bind to p53 at the same binding sites to negatively control the p53 concentration in cells [27, 28]. To identify the binding patterns of p53 with MDM2 and MDMX, we solvated the p53–MDM2 and p53–MDMX complexes respectively to perform MD simulations. After a 50-ns MD simulation, we obtained the stable complexes of the N-terminal helix of the p53-binding sites of MDM2/MDMX, as shown in Figs. 2(a), (b), and (c). The superposition of stable complex structures of p53–MDM2 and p53–MDMX indicated that the binding patterns of p53 to MDM2 and MDMX are the same, even though some differences in their sequences and structures were detected.

Analysis of the structures of the p53–MDM2 and p53–MDMX complexes showed that hydrophobic and hydrogen bond interactions are important for the binding of p53 to MDM2/MDMX, corroborating previous findings [1, 4, 8, 30–33]. The hydrophobic surfaces of the p53-binding sites on MDM2 and MDMX are sim-

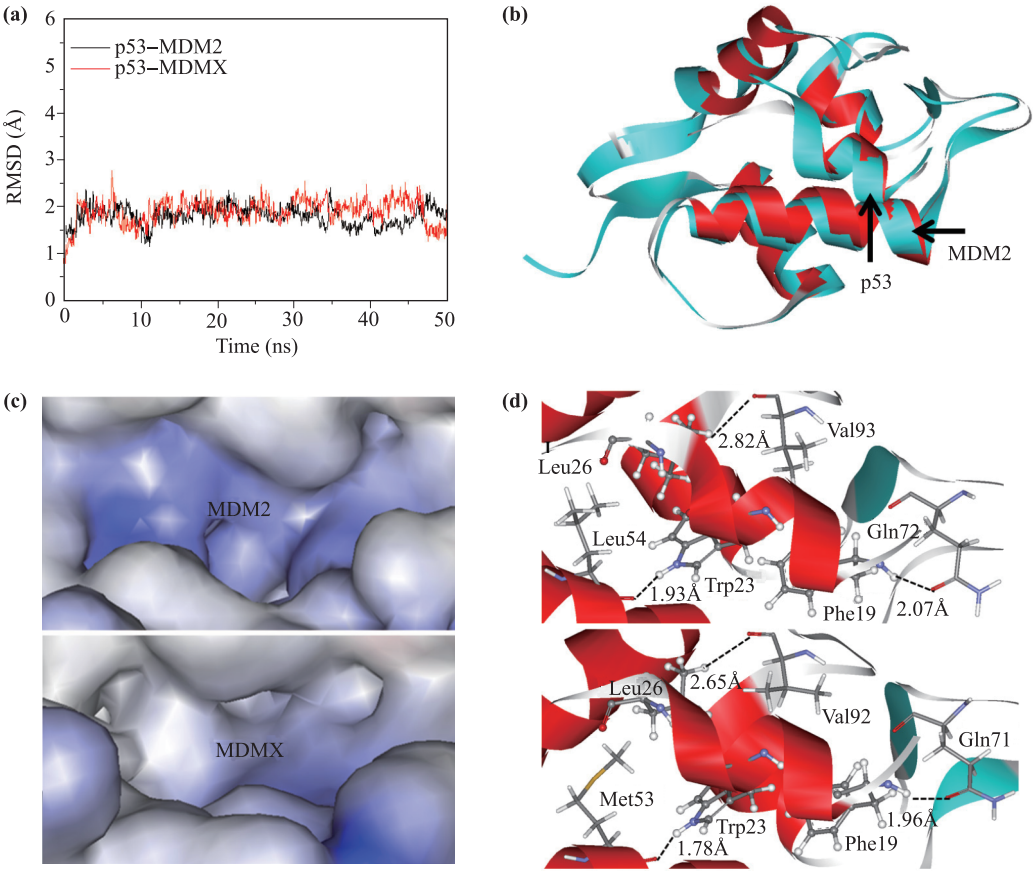

Fig. 2 Simulation results of p53–MDM2 and p53–MDMX complexes. **(a)** RMSD values of the protein–protein distance in p53–MDM2 and p53–MDMX complexes relative to their initial structures. After 5 ns, the two systems reached a stable state. **(b)** Superposition of the stable p53–MDM2 (red) and p53–MDMX (blue) complex structures. **(c)** The hydrophobic surface of the p53-binding sites of MDM2 and MDMX. The volume of this site on MDM2 is slightly smaller than that on MDMX. **(d)** The three hydrogen bonds formed between Phe19, Trp23, and Leu26 of p53 protein and Gln72/Gln71, Leu54/Met53, and Val93/Val92 of MDM2/MDMX, respectively. The distances between the O and H atoms of these hydrogen bonds are explicitly shown.

ilar, as shown in Fig. 2(c). There are three hydrogen bonds formed between Phe19, Trp23, and Leu26 of p53 and Gln72/Gln71, Leu54/Met53, and Val93/Val92 of MDM2/MDMX, as shown in Fig. 2(d). For the p53–MDM2 complex, these three hydrogen bonds are formed by the hydrogen atom HE1 of residue Trp23 of p53 and the oxygen atom O of residue Leu54 of MDM2 (HB1), the hydrogen atom HZ of residue Phe19 of p53 and the oxygen atom OE1 of residue Gln72 of MDM2 (HB2), and the hydrogen atom H15 of residue Leu26 of p53 and the oxygen atom O of Val93 of MDM2 (HB3), respectively. The most important and stable of these three hydrogen bonds is that formed between Trp23 of p53 and Leu54/Met53 of MDM2/MDMX. The same conclusion has been reported in previous studies [5, 29]. The other two hydrogen bonds are not particularly stable since they are located at the margin of the complex models and are therefore exposed to surrounding water molecules.

It is worthwhile to note that our MD simulation has realized the situation in which the Phe19, Trp23, and Leu26 residues of p53 insert into the hydrophobic cavity in MDM2 to form hydrophobic interactions [Fig. 2(b)]. The total energy of the three hydrogen bonds was calculated to be −12.68 kcal/mol. This finding reproduces the experimental observations and theoretical analyses reported in various studies [1, 4, 5, 8, 11, 29–33].

3.3 Binding of Nutlin3 with MDMX and MDM2 proteins

We further analyzed the binding characteristics of Nutlin3 with MDMX and MDM2 proteins. As described in Section 3.2, we solvated the Nutlin3–MDM2 and Nutlin3–MDMX complexes to perform the MD simulations. To check the stability of these two systems, we calculated the root mean squared deviation (RMSD) of the distance between Nutlin3 and MDM2, as well as that between Nutlin3 and MDMX. As shown in Fig. 3, the MDM2–Nutlin3 complex was fully stabilized after ∼5 ns and reached a nearly stationary state. However, the MDMX–Nutlin3 complex seemed to be loosely combined and could not become stable. This observation is consistent with the results of segmental mutagenesis experiments [12]. The snapshots of MDMX–Nutlin3 complex conformations at various simulation time points in the trajectory are shown in Fig. 4, which confirmed that Nutlin3 cannot bind to MDMX stably.

We here note that in Fig. 4 there is a large amplitude

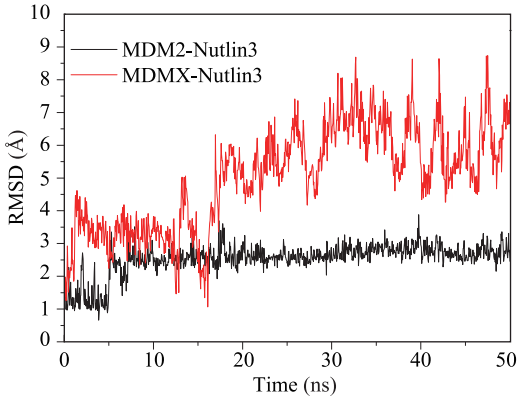

Fig. 3 RMSD values of the distance between Nutlin3 and MDM2 (black), and between Nutlin3 and MDMX (red). The MDM2–Nutlin3 complex reached a stable state after 5 ns. However, the MDMX–Nutlin3 complex could not reach a stable structure.

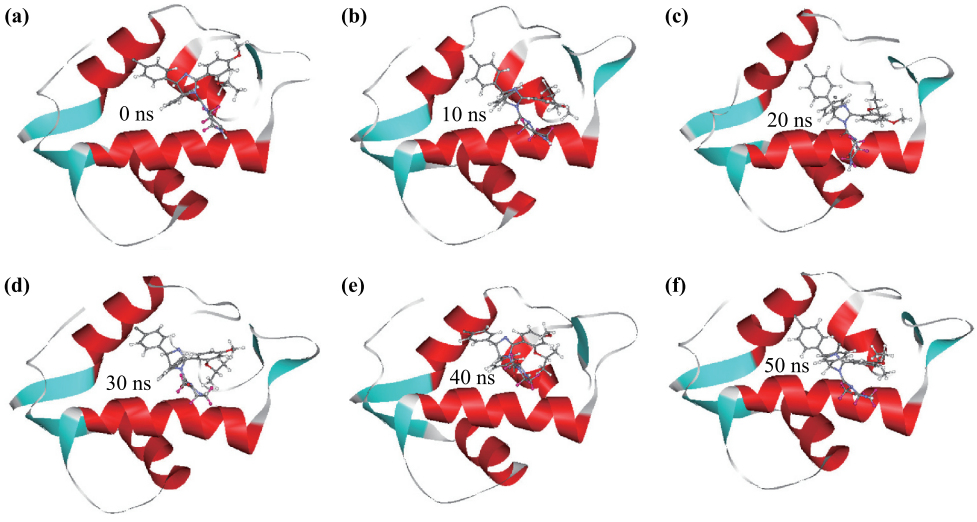

Fig. 4 Snapshots of the MDMX–Nutlin3 complex at 0 **(a)**, 10 **(b)**, 20 **(c)**, 30 **(d)**, 40 **(e)**, and 50 **(f)** ns in the MD simulation. The Nutlin3 molecule could bind to the p53-binding site of MDMX but was highly flexible.

motion of helix H3. In Figs. 4(c) and (d), H3 is not detected as a standard helix structure. H3 is located at the bottom of the binding pocket of MDMX and effectively serves to protect the interactions between p53 and Nutlin3 and the binding pocket of MDMX. However, H3 itself is not particularly stable because it is exposed to the surrounding water molecules. Two hydrogen bonds (Asp79–Glu83 and Leu80–Leu84) that maintain the helix structure showed fluctuation between the forming and opening states. In this way, H3 exhibited large amplitude motions in our simulation.

To more deeply understand the above results from an atomistic level, we conducted a further step to analyze the interactions of Nutlin3 with MDM2 and MDMX and the dynamical evolution of their complex structures. Since the hydrogen bonding site of Nutlin3 is limited, Nutlin3 could not form a stable hydrogen bond with MDM2 or MDMX in our MD simulations. Therefore, we propose that the different binding behavior of Nutlin3 to MDM2 and MDMX is caused by steric differences between MDM2 and MDMX underlying the sequence differences between these two proteins. We superimposed the initial MDM2–Nutlin3 and MDMX–Nutlin3 complexes (at the moment just after the 30 000-steps energy minimization) in the MD simulation to analyze their conformational differences, as shown in Fig. 5. Based on this analysis, we here summarize the likely reasons for why Nutlin3 cannot bind to MDMX stably as the following three aspects.

First, in our simulation models, MDM2 showed an extra α helix (helix H1 from that in Fig. 1), formed by residues Gln18 to Ala21 (Fig. 5). This α helix effectively confined the movement of the Nutlin3 molecule. However, the model of the MDMX–Nutlin3 complex lacked this α helical structure. We cannot confirm whether or not this α helix exists in the natural environment because the crystal structure of the MDMX–Nutlin3 complex is lacking. To estimate the function of this extra α helix in the binding of Nutlin3 to MDM2 or MDMX protein, we performed two MD simulations for (i) the MDM2–Nutlin3 complex with this extra α helix is deleted, and (ii) the MDMX–Nutlin3 complex harboring this extra α helix. The RMSD values of Nutlin3 in these two simulations are shown in Fig. 6. The results clearly showed that when the extra α helix is lacking, the MDM2–Nutlin3 complex becomes relatively unstable compared with that in the presence of the extra α helix. Even in this case, such unstable binding of Nutlin3 to MDM2 is much more stable than the binding of Nutlin3 to MDMX without the extra α helix. When this helix exists, Nutlin3 could bind to MDMX stably. These results convincingly suggest that the extra α helix formed by residues Gln18 to Ala21 play a crucial role in stabilizing the Nutlin3 molecule during its binding to MDM2 or MDMX protein.

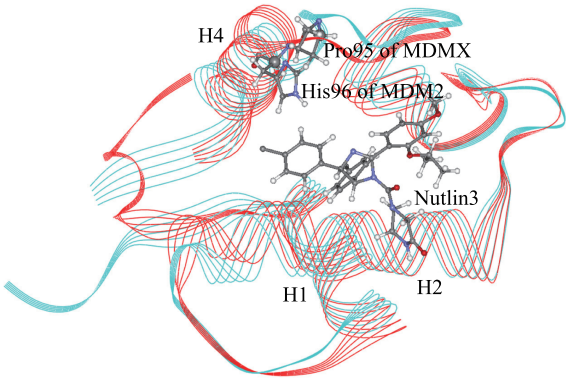

Fig. 5 Superposition of stable MDM2–Nutlin3 (red) and MDMX–Nutlin3 (blue) complex structures. The H2 and H4 helices of MDM2 are closer to each other relative to those of MDMX upon binding with Nutlin3, which results in different binding volumes of MDM2 and MDMX. The His96 residue of MDM2, corresponding to Pro95 of MDMX, covers Nutlin3 in the binding state, which enhances the stability of the inhibitor in the binding site of MDM2.

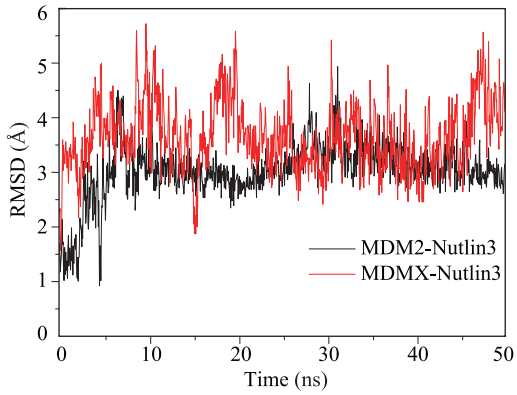

Fig. 6 RMSD values of Nutlin3 in the MDM2–Nutlin3 complex without (black) and with (red) the extra α helix.

Second, the volumes of the Nutlin3-binding sites of MDM2 and MDMX change upon binding with Nutlin3. As discussed in Section 3.1, the hydrophobic surfaces of MDM2 and MDMX are similar [Fig. 2(c)]. However, as seen in Fig. 5, the α helices H2 and H4, which form the p53- and inhibitor-binding sites of both MDM2 and MDMX, change upon Nutlin3 binding. This difference results in a larger volume of the inhibitor-binding site of MDMX than that of MDM2, so that Nutlin3 cannot bind tightly to MDMX. This conformational difference between H2 and H4 of MDM2 and MDMX arises from the sequence differences of the two ends of the H2 and H4 regions between the proteins.

Third, when focusing on individual residues, we found that certain sequence differences between MDM2 and MDMX also contribute to the different binding behav-

ior of Nutlin3 to MDM2 and MDMX. The 96th site of MDM2 is histidine and the corresponding site of MDMX is proline. The long side chain of His96 in MDM2 can form a stacking interaction with Nutlin3, which confines the movement of the Nutlin3 molecule to further enhance its stability when binding to MDM2 (Fig. 3). However, Pro95 in MDMX cannot form such interactions with Nutlin3.

The following conclusions can be reached based on the findings summarized above. There are various structural differences between the Nutlin3–MDM2 and Nutlin3–MDMX complex structures that result in the different binding characteristics of Nutlin3 to MDM2 and MDMX. Such sequence-based structural differences are in three α helices (H1, H2, and H4) and one amino acid site (His96 of MDM2 and Pro95 of MDMX). Therefore, these differences between MDM2 and MDMX should be sufficiently considered in the design of dual inhibitors of MDM2 and MDMX to enhance the stability of inhibitors at the binding site of MDMX.

3.4 Disruption of the MDM2–p53 interaction with small molecules

From a static-binding point of view, small molecules such as Nutlins can be used to displace recombinant p53 protein from its complex with MDM2 as shown in Fig. 7. However, it is still unclear how such small molecules dynamically realize the disruption of the MDM2–p53 interaction and the replacement of p53 from the complex *in vivo*, because, initially, the inhibitors are quite far away from the MDM2–p53 complex in a water environment. This is an important issue to consider when trying to understand the action principle of inhibitors.

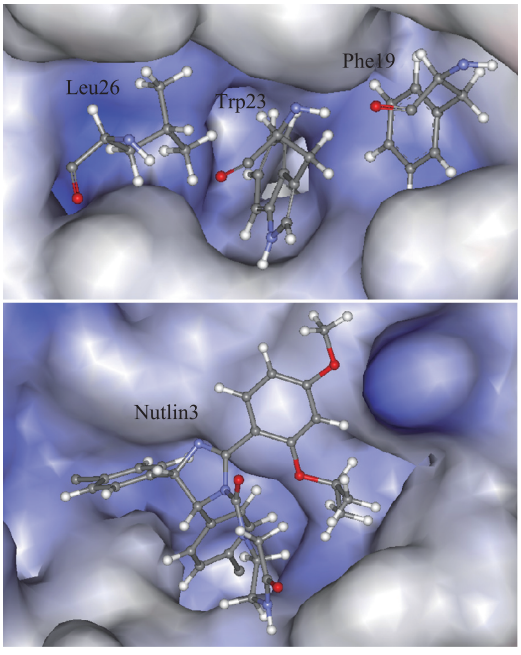

Fig. 7 p53 and Nutlin3 binding to the extended hydrophobic cleft on the N-terminus of MDM2.

In general, the process of disruption and replacement is dynamic and is affected by the complex biological environment. To investigate whether Nutlin3 can disturb the interaction between p53 and MDM2 and compete with p53 for binding to the extended hydrophobic cleft on the N-terminus of MDM2, we set the Nutlin3 molecule far away from the p53–MDM2 complex and ran the simulation for 180 ns. The whole system was set in a water environment with temperature 310 K.

Five dynamical snapshots of the system are shown in Fig. 8. Initially, at 0 ns, p53 and MDM2 could bind stably, and Nutlin3 was far away from the complex. As mentioned above, in this initial case, there are three hydrogen bonds: HB1, HB2, and HB3, among which HB1 is the most stable. The interactions between p53 and MDM2 in this initial snapshot were the same as those detected in the aforementioned simulations. At 50 ns, the interactions between p53 and MDM2 were slightly disturbed, and Nutlin3 moved closer to the binding site of MDM2. Although HB1 was still stable, the other two hydrogen bonds (HB2 and HB3) became rather weak and then broke down. At 100 ns, even HB1 became weak. At 150 ns through to 180 ns, HB1 completely broke down and p53 moved further away from the binding cavity, compared with its position at the snapshot of 100 ns.

The results described above intuitively and dynamically reveal that Nutlin3 can destroy the interactions between p53 and MDM2, go into the binding site of MDM2, and ultimately forms a complex with MDM2 (this is expected if the MD simulation is run for long enough). This competition mechanism is the most important basis for understanding the inhibition mechanism of small-molecule inhibitors for development of an effective cancer therapeutic strategy. Therefore, this microscopic study may establish a linkage with the macroscopic investigations of the p53 pathway and their stochastic dynamics [34–36]; for example, by providing the important parameters such as reaction rate constants and time scales.

4 Concluding remarks

In this study, we systematically investigated the structural characteristics and interaction properties of inhibitor–MDM2 and –MDMX complexes at the atomistic level, and compared the interaction of p53 with MDM2 and MDMX to explore the molecular basis of inhibition. The results revealed that the structures of MDM2 and MDMX are very similar except for some

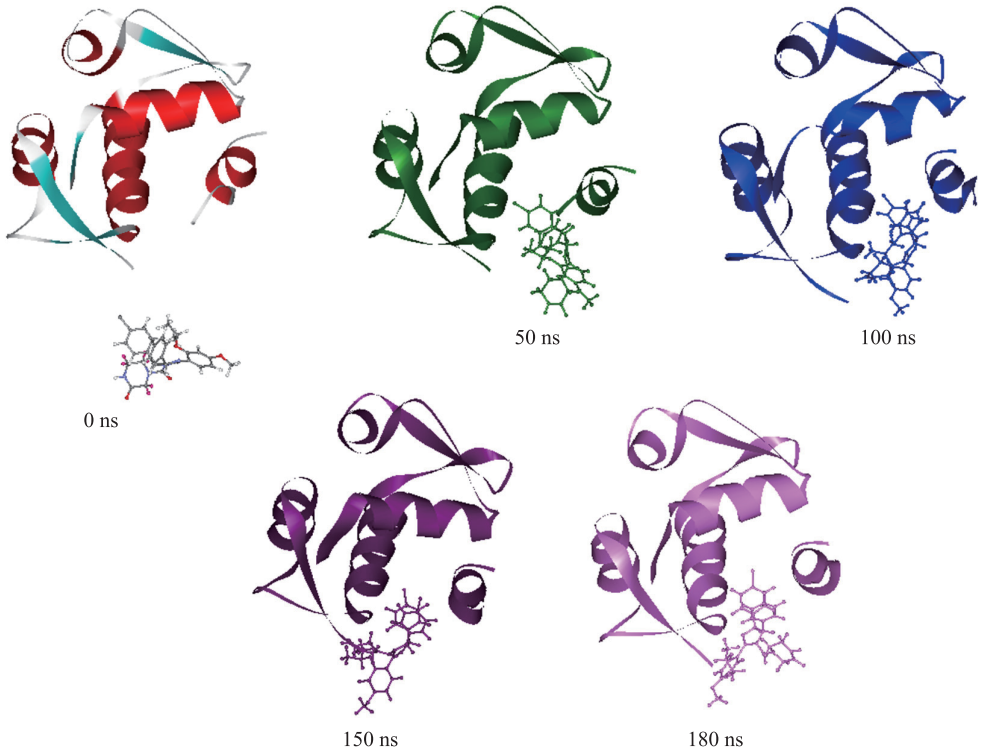

Fig. 8 Dynamical snapshots of the Nutlin3–MDM2–p53 complex at various time points of the MD simulation.

amino acid differences in two α helices, H3 and H4, of MDM2 and/or MDMX. The formation of the complexes of p53–MDMX and p53–MDM2 relies on hydrophobic and hydrogen bond interactions.

We further explored the reason for the ability of Nutlin3 to combine with MDM2 and not MDMX. We have now clarified that there are three factors influencing the binding of Nutlin3 to MDM2 and/or MDMX. First, an extra α helix at the N-terminus of MDM2 and/or MDMX is important for the tight binding of Nutlin3 to MDM2 and MDMX. Second, in the dynamical docking process of Nutlin3 to MDM2/MDMX, there are conformational changes in two α helices, H2 and H4, which result in different volumes of the inhibitor-binding sites of MDM2 and MDMX. Third, His96 in MDM2 plays an important role in the binding of Nutlin3 to MDM2.

From a dynamical point of view, we have proven that Nutlin3 can combine with MDM2 and effectively inhibit the p53–MDM2 interaction. Restoration of the activity of p53 by inhibiting the interaction of p53 with MDM2 and/or MDMX is a promising and feasible method for cancer therapy, although its true therapeutic potential is yet to be elucidated.

It should be noted that the studies reported in this paper are still at a very initial stage. The detailed information needs to be verified through further structural and dynamical investigations. To gain sufficient understanding of the inhibition mechanisms, it will be important to combine various methods such as a molecular docking method, MD simulation, free energy analysis, and even stochastic network dynamics [37]. The molecular docking method is expected to provide a reasonable initial structure of inhibitor molecules for MD simulation. Considering that biomolecules are generally highly heterogeneous, extremely dynamical, far from equilibrium, and in a complex fluctuating environment, stochastic network dynamics analyses are needed to study the p53 pathway [38]. There is no doubt that such investigations represent a challenging task, but with further efforts, we expect to realize these goals.

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