Discovery of a Nanomolar Inhibitor of the Human Murine Double Minute 2 (MDM2)−p53 Interaction through an Integrated, Virtual Database Screening Strategy

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Abstract: An integrated, virtual database screening strategy has led to 7-[anilino(phenyl)methyl]-2-methyl-8-quinolinol (**4**, NSC 66811) as a novel inhibitor of the murine double minute 2 (MDM2)−p53 interaction. This quinolinol binds to MDM2 with a K_i of 120 nM and activates p53 in cancer cells with a mechanism of action consistent with targeting the MDM2−p53 interaction. It mimics three p53 residues critical in the binding to MDM2 and represents a promising new class of non-peptide inhibitors of the MDM2−p53 interaction.

The p53 tumor suppressor is central to the regulation of cell cycle progression, DNA repair, and apoptosis[1−3] and is an attractive cancer therapeutic target because its tumor suppressor activity can be stimulated to eradicate tumor cells.[2−5] Since p53 effectively suppresses oncogenesis, it is not surprising that in approximately 50% of all human cancers its function has been nullified by deletions or mutations in the DNA-binding domain of p53.[6] In the remaining 50% of human cancers, p53 retains its wild-type form but its activity is effectively inhibited by its cellular inhibitor, the human murine double minute 2 (MDM2) oncoprotein through direct interaction with p53.[4,7] Reactivation of the p53 function by disruption of the MDM2−p53 interaction using a non-peptide small-molecule inhibitor is now recognized as a new and promising strategy for anticancer drug design.[4,5] A number of classes of non-peptide small-molecule inhibitors of the MDM2−p53 interaction have been reported recently.[5,8−14] The chemical structures of three non-peptide, potent small-molecule inhibitors of the MDM2−p53 interaction are shown in Figure 1. These include a Nutlin (compound **1**)[8], a benzodiazepine-based inhibitor (compound **2**)[10] and a spiro-oxindole-based inhibitor (compund **3**).[9]

Several approaches have been employed to identify non-peptide small-molecule lead compounds targeting the MDM2−p53 interaction.[4,5,8−13] Initial leads that led to the design of the Nutlins[8] and benzodiazepine-based inhibitors of the MDM2−p53 interaction[10] were identified by experimental high-throughput screening, and a computational pharmacophore-based approach has been used to discover a non-peptide small-molecule inhibitor.[13] The spiro-oxindole-based inhibitors of the MDM2−p53 interaction recently reported by our laboratory were designed by a de novo computational structure-based approach.[9] In this study, we present the discovery through computational database screening of a non-peptide small-molecule inhibitor of the MDM2−p53 interaction with nanomolar binding affinity.

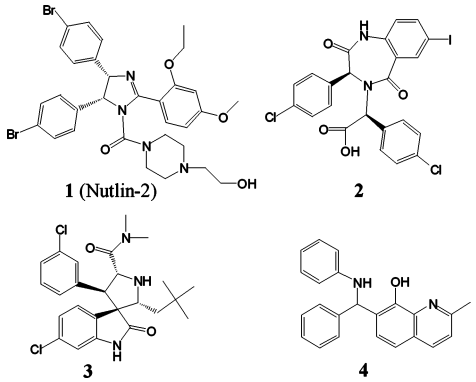

Figure 1. Chemical structures of three previously reported potent inhibitors of the MDM2−p53 interaction and a new inhibitor (**4**) discovered from the current study.

A crystallography study showed that the interaction between p53 and MDM2 involves a short helix formed by the N-terminus of p53 and a small, deep hydrophobic cleft in MDM2.[17] This cleft is an attractive site for the binding of small-molecule inhibitors that can block the MDM2−p53 interaction,[4] and it is the target site for our database screening.

Two popular computational 3D database screening approaches have been employed in drug lead discovery: pharmacophore and structure-based searching approaches. In pharmacophore searching, a pharmacophore model is defined consisting of chemical groups critical for ligand binding to a target protein with their 3D geometrical relationship. A computational search is then performed to identify compounds ("hits") whose 3D structures meet the requirements specified in the pharmacophore model. The advantages of pharmacophore searching include the relatively short computing time necessary to search a large chemical database and fast elimination of compounds that lack critical binding elements. The shortcomings are that this approach is qualitative in nature and many of the "hits" are inactive because of, for example, their lack of spatial complementarity which results in inability to interact with the target protein effectively. In structure-based database searching, each compound in a chemical database is computationally docked into the binding site in the target protein and its binding affinity is then assessed using a scoring function. One major advantage of structure-based searching is that it can quantitatively assess the binding affinity for a compound to its target protein. However, for a database containing hundreds of thousands of compounds, the computational time available for each compound is very limited for practical reasons. Such limited computing time often results in inaccurate prediction of the binding models, especially for flexible compounds. In addition, current scoring functions have much room for improvement in their accuracy of binding affinity prediction.[15]

To take advantage of these two complementary computational screening approaches and overcome their limitations, we have employed an integrated database screening strategy in this study (Chart 1 and Supporting Information). First, we have developed a simple pharmacophore model (Figure 2) based on the crystal structure of the p53 peptide complexed with MDM2 and several known non-peptide small-molecule inhibitors. This model was used to perform a pharmacophore search to identify "hits" that satisfy the chemical and the geometrical requirements (Chart

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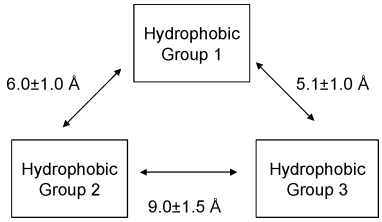
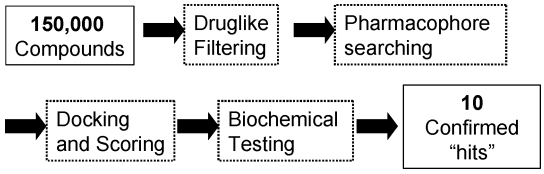


Figure 2. Proposed simple pharmacophore model based on the crystal structure of p53 peptide in complex with MDM2 and several known, non-peptide, small-molecule inhibitors of the MDM2−p53 interaction.

Chart 1. Integrated Computational Screening Strategy for the Discovery of Inhibitors of the MDM2−p53 Interaction



1). Finally, structure-based searching was used to dock each "hit" to the p53 binding site in MDM2 and to rank the binding affinities. Because pharmacophore searching dramatically reduces the number of compounds for docking studies, one can employ more time-consuming computational docking procedures to obtain more accurate binding models for ligands.

We chose to screen the National Cancer Institute (NCI)'s 3D database[18,19] on the basis of the following considerations. First, this 3D database contains not only structurally diverse synthetic compounds collected from many laboratories around the world but also a large number of natural products.[18,19] The value of such structural diversity in drug discovery is increasingly being appreciated. Second, the NCI database contains a large number of known drugs, as well as analogues of known drugs and other non-peptide druglike molecules, and provides an opportunity to discover high-quality lead compounds.[19] One caution is that chemical structures for some compounds in the NCI database may be incorrect, and for this reason, once a lead compound is identified, its chemical structure needs to be carefully verified and confirmed. We have employed a web-based, flexible pharmacophore searching tool developed in our laboratory[20] for pharmacophore searching and the GOLD program[21,22] for structure-based searching. Since the number of compounds is dramatically reduced by first applying pharmacophore screening, we were able to use a set of docking parameters in the GOLD program that is 6 times more computationally demanding but achieves a higher degree of sampling than the default GOLD database screening parameters (Supporting Information).

Although the entire publicly accessible NCI database[18,19] contains approximately 250 000 compounds, we focused our computational screening on the approximately 150 000 compounds for which chemical samples were available in sufficient quantity for biological testing when the 3D database was first built in 1992.[18] These 150 000 compounds were further filtered using a number of simple criteria (Supporting Information) to remove approximately 40 000 nondruglike molecules and provide our working database of 110 000 compounds.

Superposition of the experimentally determined binding poses for the p53 peptide,[17] **1**,[8] and **2**[10] and the predicted binding pose for **3** led to the identification of three hydrophobic groups occupying the Phe19, Trp23, and Leu26 subpockets of MDM2 as the common features among these peptide or non-peptide inhibitors. A pharmacophore model was developed to capture these three common binding features (Figure 2), with a set of distance constraints to define the geometrical relationships between these groups. Pharmacophore searching of the 110 000 compounds led to identification of 2599 "hits" that met the requirements specified in the pharmacophore model. Structure-based screening of these 2599 "hits" was performed using GOLD with the ChemScore fitness function. We have previously shown[16] that in terms of its ability to rank compounds for their relative binding affinities the X-score scoring function[15] is superior to most of the other scoring functions, and accordingly, we also employed X-score to rank those 2599 compounds on the basis of their predicted binding poses by GOLD.

The top 200 compounds ranked by ChemScore or X-score were selected for further examination. There were 46 overlaps between the top 200 compounds ranked by ChemScore and X-score. These 354 nonredundant compounds were considered as potential inhibitors of the MDM2−p53 interaction. We have performed visual inspection to confirm that each of these 354 compounds mimics the key hydrophobic interactions between p53 and MDM2 observed in the crystal structure and that it is spatially complementary with MDM2 based on the predicted binding models by GOLD. To date, chemical samples for 103 compounds have been requested from the NCI and 67 of them have been received and tested.

We included three known potent inhibitors of the MDM2−p53 interaction with diverse chemical structures (compounds **1**, **2** and **3** in Figure 1) as positive controls. Each of these three representative inhibitors meets the chemical and geometrical requirements specified in our pharmacophore model. We then performed docking studies using the same GOLD parameters we used for our database screening to determine if, for these compounds, GOLD can predict the binding models obtained using X-ray crystallography[8,10] or extensive computational modeling.[9] It was found that GOLD fails to reproduce the experimentally determined binding pose for **1** in its crystal structure complexed with MDM2,[8] but the binding pose predicted by GOLD for **2** is in good agreement with that found in the experimentally determined crystal structure,[10] with an overall root-mean-square deviation of 1.9 Å for non-hydrogen atoms (Supporting Information). The binding pose for **3** predicted by GOLD is also in excellent agreement with that developed by our previous modeling studies.[9] Thus, GOLD was able to predict the binding models for 2 out of 3 compounds using the database screening parameters we chose in our study. Examination of the ranks for these three positive controls showed that **2** and **3** were among the top 200 predicted by ChemScore. The nutlin (**1**), whose binding pose was not predicted correctly by GOLD, was ranked only among the top 1000 compounds.

Using a quantitative and sensitive fluorescence-polarization-based (FP-based) competitive binding assay developed in our laboratory (Supporting Information), we have evaluated these 67 compounds for their ability to display a fluorescently tagged p53-based peptide from the MDM2 protein. Ten compounds were found to have a K_i of less than 10 μM in this competitive binding assay (data not shown).

Among these 10 active compounds, 7-[anilino(phenyl)-methyl]-2-methyl-8-quinolinol (**4**, NSC 66811) has the highest binding affinity with a K_i of 120 nM (Figure 3). In direct comparison, nutlin-3[8] and **3**, two known potent inhibitors of the MDM2−p53 interaction, have K_i values of 36 and 84 nM, respectively, in this competitive binding assay (Figure 3). The natural p53 peptide (residue 13−29) has a K_i of 6670 nM in this assay. Compound **4** is thus 3.3 and 1.4 times less potent

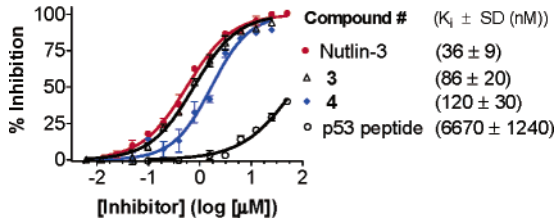


Figure 3. Competitive binding curves of **4**, two known inhibitors, and a natural p53 peptide to MDM2 as determined using a competitive FP-based binding assay. Three to five independent experiments were performed for each compound.

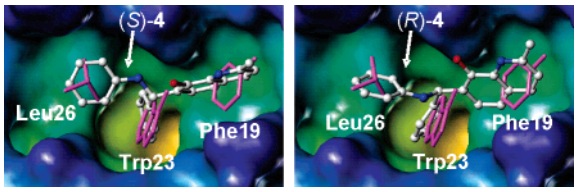


Figure 4. Predicted binding models for (*S*)- and (*R*)-**4** to MDM2 and superposition onto the crystal structure of p53 peptide in complex with MDM2. Side chains of three key p53 binding residues (Phe19, Trp23, and Leu26) are shown.

than nutlin-3 and **3**, respectively, but is 56 times more potent than the natural p53 peptide. We have shown by NMR and mass spectrometry that the chemical structure for **4** in the NCI database is indeed correct (Supporting Information). Significantly, **4** has a chemical structure that is completely different from the structures of the nutlins,[8] our spiro-oxindoles,[9] and other known inhibitors of the MDM2−p53 interaction.[5]

To understand the structural basis of the binding of **4** to MDM2, we have performed extensive computational docking studies using GOLD. Since **4** has a chiral center and the chemical sample from the NCI is racemic, we have docked both the (*S*)-**4** and (*R*)-**4** into the binding site of MDM2 and compared the predicted binding models to that of p53 to MDM2. The X-ray structure of p53 in complex with MDM2 shows that their interaction is mediated mainly by three key hydrophobic p53 residues, namely, Phe19, Trp23, and Leu26. The docking results showed that the (*S*)- and (*R*)-stereoisomers of **4** nicely mimic the three key p53 residues (Phe19, Trp26, and Leu26) for interaction with MDM2 (Figure 4). The predicted scores using ChemScore and X-score suggested that the (*S*)-isomer binds slightly more strongly than the (*R*)-isomer. But further studies are needed to determine which isomer is the more active form for binding to MDM2.

A potent, cell-permeable, small-molecule inhibitor of the MDM2−p53 interaction is expected to activate p53 function, resulting in an increase in the levels of p53 protein in cells with wild-type p53 but not in cells with mutated or deleted p53. In addition, functional activation of p53 should lead to induction of p21 cyclin-dependent kinase inhibitor 1 (*p21*$^{cip1/waf}$) and *MDM2*, two p53 targeted genes, and result in an increase in the levels of p21$^{cip1/waf}$ and MDM2 proteins.[8] To test these predictions, we have examined by Western blot analysis the levels of p53, MDM2, and p21$^{cip1/waf}$ proteins in the HCT-116 human colon cancer cell line with wild-type p53 and the corresponding isogenic p53 knock-out cell line when treated with **4** (Figure 5). As can be seen, **4** dose-dependently induces the accumulation of p53, MDM2, and p21$^{cip1/waf}$ proteins in the HCT-116 human colon cancer cell line with wild-type p53. Importantly, **4** has no effect on the levels for p53, MDM2, and p21$^{cip1/waf}$ protein in the isogenic HCT-116 p53$^{-/-}$ cell line. These data indicate that the accumulation of MDM2 and p21$^{cip1/waf}$ proteins in the HCT-116 p53$^{+/+}$ cell line treated with

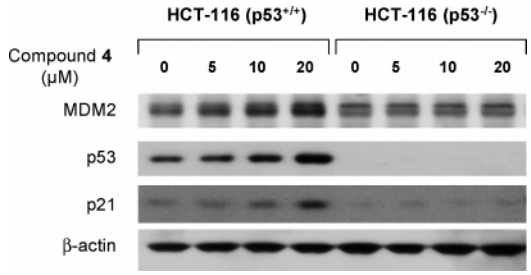


Figure 5. Western blot analysis of p53 protein and two p53 targeted gene products of MDM2 and p21$^{cip1/waf}$ in HCT-116 colon cancer cell line with wild-type p53 (p53$^{+/+}$) and the corresponding isogenic p53 knock-out (p53$^{-/-}$) cell line. Cells were treated for 48 h by **4**, and protein levels were probed by antibodies against p53, MDM2, and p21$^{cip1/waf}$ proteins.

4 is due to the functional activation of p53. Importantly, unlike chemotherapeutic agents such as doxorubicin but consistent with its mode of action in blocking the MDM2−p53 interaction, **4** does not cause the phosphorylation of p53 at its serine-15 residue (Supporting Information).

In summary, using an integrated computational database screening strategy, we have discovered 7-[anilino(phenyl)-methyl]-2-methyl-8-quinolinol (**4**) as a structurally novel, potent, non-peptide, druglike, small-molecule inhibitor of the MDM2−p53 interaction. Compound **4** has a binding affinity of 120 nM binding to MDM2 and is 56 times more potent than the natural p53 peptide (residues 13−29). Our docking studies suggest that **4** mimics Phe19, Trp23, and Leu26, three key p53 residues mediating the interaction with MDM2. Compound **4** activates p53 function in the HCT-116 cancer cell line with wild-type p53 but not in the corresponding isogenic p53 knock-out isogenic cell line. Compound **4** therefore represents a novel class of inhibitors of the MDM2−p53 interaction and is a promising lead compound for further optimization. This study shows that our integrated database screening strategy is effective in the discovery of potent, non-peptide, small-molecule inhibitors of the MDM2−p53 protein−protein interaction.

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Supporting Information Available: Information on database screening and computational docking, the fluorescence-polarization-based competitive binding assay, cellular experiments to determine the protein levels of p53, MDM2, and p21$^{cip1/waf}$ and phosphorylation of p53 at its serine-15 residue in HCT-116 p53$^{+/+}$ and p53$^{-/-}$ cell lines, and analysis of the chemical structure of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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