Top Med Chem 8: 57–80
DOI: 10.1007/978-3-642-28965-1_2

Targeting the MDM2-p53 Protein-Protein Interaction for New Cancer Therapeutics

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Contents

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Abstract The p53 tumor suppressor protein is a transcriptional factor that plays a key role in regulation of several cellular processes, including the cell cycle, apoptosis, DNA repair, and angiogenesis. The murine double minute 2 (MDM2) protein is the primary cellular inhibitor of p53, functioning through direct interaction with p53. Design of non-peptide, small-molecule inhibitors that block the MDM2-p53 interaction has been sought as an attractive strategy to activate p53 for the treatment of cancer and other human diseases. Major advances have been made in the design of small-molecule inhibitors of the MDM2-p53 interaction in recent years, and several compounds have moved into advanced preclinical development or clinical trials. In this chapter, we will highlight these advances in the design and development of MDM2 inhibitors, and discuss lessons learned from these efforts.

Keywords HDM2 • Inhibitors • MDM2 • Protein-protein interactions

1 Introduction

1.1 The p53 Tumor Suppressor and Its Primary Cellular Inhibitor MDM2 Protein

Tumor suppressor p53 is a transcriptional factor that plays a pivotal role in the regulation of the cell cycle, apoptosis, DNA repair, senescence, angiogenesis, cellular metabolism, and innate immunity [1–3]. The p53 protein was identified in 1979 [4–6], and its gene, called *TP53*, was cloned in 1983 [7]. Consistent with its role as a prominent tumor suppressor, p53 is functionally inactivated by mutation or deletion in nearly 50% of human cancers [8]. In cancers with wild-type p53, its function is effectively inhibited by its primary cellular inhibitor, the murine double minute 2 (MDM2; HDM2 in humans) protein. MDM2 is an oncoprotein that was initially discovered by its overexpression in a spontaneously transformed mouse cell line [8–12]. The cellular levels of p53 and MDM2 are mutually regulated through an autoregulatory feedback loop (Fig. 1). As a transcription factor, p53 binds to the promoter and increases expression of the *Mdm2* gene. In turn, MDM2 protein directly binds to p53 and inhibits the activity of p53 through multiple mechanisms: MDM2 (1) directly inhibits the transactivation function of p53, (2) exports p53 out of the nucleus, and (3) promotes proteosome-mediated degradation of p53 through its E3 ubiquitin ligase activity [13–15]. The physiological relevance of this regulatory loop was demonstrated by the genetic evidence that embryonic lethality of *Mdm2*-null mice can be successfully rescued by the simultaneous deletion of the p53 gene [16, 17].

Deregulation of the MDM2/p53 balance leads to malignant transformation of normal cells. For example, overexpression of MDM2 provides cells a growth advantage, promotes tumorigenesis, and correlates with a worse clinical prognosis and poor response to cancer therapy [18–24]. A variety of mechanisms, such as

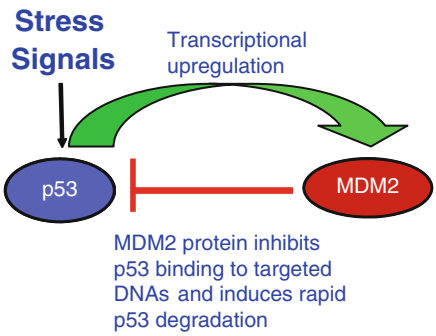

Fig. 1 Autoregulatory loop between p53 and MDM2. Upon activation, p53 transcribes the MDM2 gene and increases the levels of MDM2 protein. In turn, MDM2 protein binds to p53, inhibiting its binding to targeted DNAs and inducing p53 degradation

amplification of the *MDM2* gene, single nucleotide polymorphism at nucleotide 309 (SNP309) in its gene promoter, enhanced transcription, and increased translation account for MDM2 overproduction [18, 24–26]. Genetic studies in mice have revealed that overexpression of MDM2 at an early stage of differentiation neutralizes p53 tumor suppressor function and predisposes mice to tumorigenesis [27]. As with the human inherited cancer predisposition Li-Fraumeni syndrome, mice lacking p53 develop normally, but are predisposed to develop a variety of tumors [28]. Analysis of 28 different types of human cancers in nearly 4,000 human tumor samples showed that MDM2 is amplified in 7% of human cancers and MDM2 overexpression by amplification and p53 mutations are largely mutually exclusive [22]. Collectively, these data support the notion that MDM2 is a chief, though not the only, regulator of p53 function.

1.2 Blocking the Interaction of MDM2 and p53 as a New Cancer Therapeutic Strategy

Because of the critical inhibitory role of MDM2 on p53, blocking the interaction of MDM2 and p53 has been proposed as a potential cancer therapeutic strategy. The MDM2-p53 interaction was mapped to the first ~120 amino acid residues at the N-terminus of MDM2 and to the N-terminus of the transactivation domain of p53 [29, 30]. The high-resolution crystal structures of human and *Xenopus laevis* MDM2 complexed with short p53 peptides (residues 15–29) [31] were solved in 1996, and have provided atomic details of the interaction. These structures show that the MDM2-p53 interaction is mediated by a well-defined hydrophobic surface pocket in MDM2 and three key hydrophobic residues in p53, namely Phe19, Trp23, and Leu26 (Fig. 2). The relatively compact binding pocket in MDM2 makes it appear possible to design non-peptide, drug-like small-molecule inhibitors to block this interaction as a means to reactivate p53 in cells harboring wild-type p53.

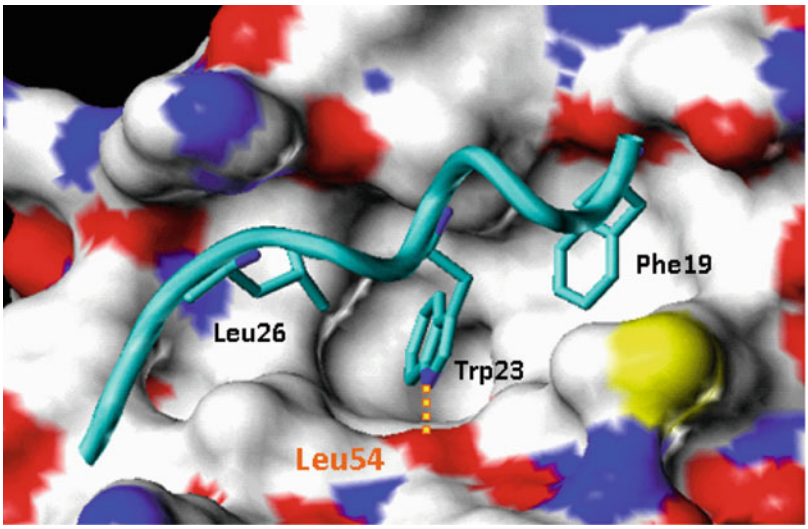

Fig. 2 Crystal structure of MDM2 protein complexed with p53 peptide (residues 13–29)

1.3 Design of High-Affinity Peptide-Based Small-Molecule Inhibitors of the MDM2-p53 Interaction

Wild-type p53 peptides have modest affinity, with K_d values of ~1 μM [31]. To explore whether it was possible to design compounds with much higher binding affinities, extensive modifications have been made on p53 peptides using natural and unnatural amino acids. These efforts led to the discovery of peptidomimetics that bind to MDM2 with K_d values of 1 nM, 1,000-fold more potent than wild-type p53 peptides [32]. The data have provided direct evidence that the MDM2 binding pocket is indeed a feasible target for the design of high-affinity small-molecule inhibitors. Furthermore, the structure-activity relationships obtained from peptidomimetics have proven to be useful in guiding the design of non-peptide, small-molecule inhibitors of the MDM2-p53 interaction.

Despite their high affinity, these peptide-based compounds were not useful for cellular studies, primarily due to their poor cell permeability. To enhance their permeability, compounds were tethered to carrier peptides, which resulted in compounds that activated p53 function in cells at concentrations of 100–500 μM. Although such weak cellular activity clearly makes them unsuitable as potential therapeutic agents, they nevertheless provided the initial proof-of-concept that blocking the interaction between MDM2 and p53 can indeed be an effective means of activating p53.

2 Different Approaches Toward the Discovery of Non-peptide, Small-Molecule Inhibitors of the MDM2-p53 Interaction

One critical step in the design and development of non-peptide, small-molecule inhibitors of the MDM2-p53 interaction is the discovery of novel lead compounds. Different approaches have been employed to discover such lead compounds. These include experimental screening of chemical libraries, computational three-dimensional (3D) database screening of large chemical libraries, and structure-based *de novo* design. Below we review these different approaches.

2.1 Experimental Screening of Small-Molecule Libraries

The Nutlins, discovered by Vassilev and colleagues at Hoffman-La Roche [33], are probably the first potent and specific MDM2 inhibitors. The Nutlins have a *cis*-imidazoline core structure and the initial lead compound was discovered by experimental screening of a diverse library of synthetic compounds using a Biacore surface plasmon resonance assay, although no details have been provided. Extensive chemical modifications of the initial lead compound have ultimately yielded a class of potent small-molecule MDM2 inhibitors. Nutlin-1 and Nutlin-2 are racemic compounds and Nutlin-3a is an active enantiomer (Fig. 3). Nutlins 1–3 block the MDM2-p53 protein-protein interaction with IC_{50} values of 260, 140, and 90 nM, respectively.

Benzodiazepinedione-based MDM2 inhibitors were discovered in a high throughput screening of 338,000 compounds from combinatorial libraries using ThermoFluor microcalorimetry technology [34]. Initial screening led to identification of 1,216 compounds, including 116 benzodiazepinediones, that bound to MDM2. The affinity of selected compounds was further evaluated in an FP-based p53 peptide-displacement MDM2 binding assay. The benzodiazepinedione **1** had a K_i of 80 nM in the FP-based assay. Two additional studies have also reported the discovery and characterization of benzodiazepinedione compounds as fairly potent MDM2 inhibitors using the same technique.

A homogeneous time-resolved fluorescence (HTRF) high throughput screening of ~1.4 million internal library compounds was performed by scientists at Amgen [35]. One compound, the chromenotriazolopyrimidine **2**, was found to have an IC_{50} value of 1.2 μM in the HTRF assay. Subsequent optimization has yielded a class of fairly potent MDM2 inhibitors.

Terphenyl compounds have been put forward as bearing a scaffold capable of mimicking one face of α-helical peptides [36]. The side chains in the designed terphenyl compounds are projected in a similar way to i, $i + 4$, and $i + 7$ residues in α-helical peptides. Since the interaction between p53 and MDM2 is mediated by a short α-helix from p53, it was expected that certain terphenyl compounds may also bind to MDM2. A FP-based competitive binding assay was used to evaluate the

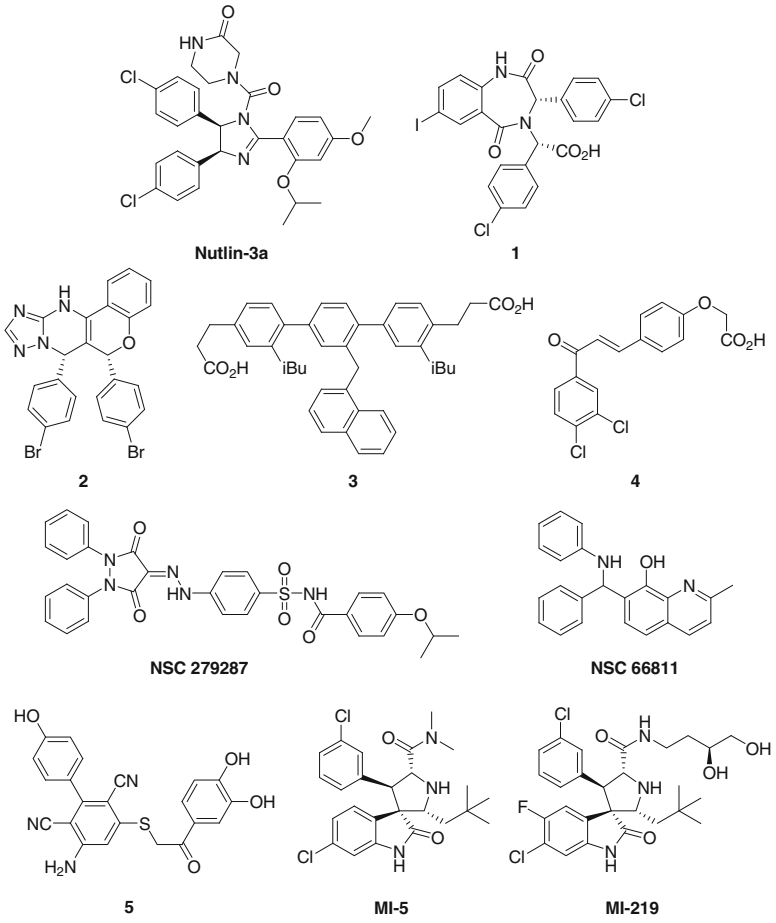

Fig. 3 Representative MDM2 inhibitors discovered using different approaches

binding affinities of 21 designed terphenyl compounds to MDM2. One such compound **3**, (Fig. 3) was found to bind to MDM2 with a K_i of 182 nM [37]. ^{15}N Heteronuclear single quantum correlation (HSQC) NMR spectroscopy further showed that this terphenyl targets the same surface area in MDM2 where p53 binds. Its specificity was evaluated for its binding to Bcl-2 and Bcl-xL proteins since Bcl-2 and Bcl-xL also bind to α-helical peptides. This terphenyl displays 82- and 14-fold selectivity over Bcl-2 and Bcl-xL, respectively, suggesting that although terphenyl compounds are general mimics of helical peptides, some can display certain selectivity for MDM2 over other proteins.

Stoll et al. [38] screened a library of 16 chalcones, which are broadly known to have anticancer properties [39], to identify compounds for disrupting the MDM2-p53 interaction. The most potent compound found, **4**, had an IC_{50} value of 49 μM.

2.2 Computational Structure-Based Screening

Galatin and Abraham were the first to employ a computational pharmacophore searching strategy to discover small-molecule inhibitors of the MDM2-p53 interaction [40]. The hydropathic interactions (HINT) program was used to generate noncovalent interaction measurements between reported p53-based peptide inhibitors and MDM2, which led to the development of a pharmacophore model [41]. Various permutations of this MDM2-binding pharmacophore model were entered as searches to obtain hit compounds from the National Cancer Institute (NCI) database [42]. These candidate compounds were tested in an in vitro MDM2-p53 protein-protein interaction assay. One compound, NSC279287 (Fig. 3), was found to disrupt the interaction between full-length MDM2 and p53 proteins with an IC_{50} value of 31.8 μM. When evaluated in a p53 reporter gene assay, only a 20% increase in p53 transcriptional function after treatment with 100 μM of the compound was observed [40]. Despite the weak affinity, this study nevertheless demonstrated that computational pharmacophore searching can be used to successfully identify small-molecule inhibitors of the MDM2-p53 interaction. No chemical modifications were reported for this class of compounds.

Subsequently, Lu et al. employed an approach combining pharmacophore and structure-based screening to search for small-molecule inhibitors of the MDM2-p53 interaction in an NCI database of 150,000 compounds [42]. A number of filters were used to remove approximately 40,000 non-druglike molecules, providing a working database of 110,000 compounds. A 3D-pharmacophore model was derived from the X-ray crystal structure of the p53 peptide complexed with MDM2, and with several known small-molecule inhibitors. The pharmacophore model consisted of three elements that mimic the three key hydrophobic binding residues in p53 (Phe19, Trp23, Leu26), together with three associated distance constraints. Pharmacophore searching of the 110,000 compounds yielded 2,599 hits. Computational docking was performed using the GOLD program [43, 44] to dock each hit to the p53-binding site in MDM2. Their binding affinities were ranked using Chemscore [45] and *X*-score [46], and the top 200 ranked compounds from each scoring function were combined, yielding 354 non-redundant compounds. A total of 67 compounds were obtained from the NCI and tested in a competitive FP-based MDM2 binding assay. Ten compounds were found to have K_i values of <10 μM. NSC66811 was the most potent compound with a K_i of 120 nM. NSC66811 activated p53 in the LNCaP prostate cancer cell line with wild-type p53 in a dose-dependent manner, providing evidence for its cellular mechanism of action. No chemical modifications were reported for any of these compounds.

Another computational search was performed using a pharmacophore model based on the crystal structure of MDM2 with incorporation of protein flexibility assessed using molecular dynamics simulation [47]. Pharmacophore searching was performed on a database of 35,000 synthetic compounds, followed by evaluation of 24 hits in a fluorescence-polarization MDM2 binding assay that led to the discovery of five non-peptidic, small-molecule MDM2 inhibitors with new scaffolds.

The most potent compound, **5** (Fig. 3), had a K_i of 110 nM to MDM2. Their cellular activity and mechanism of action have not been reported.

2.3 Structure-Based De Novo Design

Computational and experimental screening aim at identification of lead compounds from small-molecule libraries of compounds which have been synthesized previously and have been shown to be effective in the discovery of novel lead compounds as MDM2 inhibitors. However, these screening approaches have the limitation that leads are confined within existing chemical space. To overcome this limitation, the Wang laboratory at the University of Michigan employed a structure-based *de novo* design strategy to design new classes of small-molecule inhibitors to target the MDM2-p53 interaction [48, 49].

Analysis of the crystal structure of p53 complexed with MDM2 showed that the indole ring of the Trp23 residue of p53 is buried deeply inside a hydrophobic cavity in MDM2 and its NH group forms a hydrogen bond with the backbone carbonyl in MDM2. Hence, Trp 23 represents the most critical residue for binding of p53 to MDM2. In searching for a chemical moiety that can mimic the interaction between the indole group and MDM2, the oxindole ring system was found to perfectly mimic the hydrophobic and hydrogen-bonding interactions of the indole. Since many anticancer drugs are natural products or derivatives of natural products, substructure searching was performed to identify natural products that contain an oxindole ring. A number of natural alkaloids such as spirotryprostatin A and alstonisine were found to contain a spirooxindole core structure. However, computational docking of these natural alkaloids suggested that none of them could effectively interact with the MDM2 binding pocket due to steric hindrance. However, the spiro(oxindole-3,3′-pyrrolidine) core structure (hereafter called spirooxindole) emerged as the starting point for the design of a new class of MDM2 inhibitors. In the design, the oxindole closely mimics the Trp23 side chain in p53 in both hydrogen-bonding and hydrophobic interactions with MDM2. Two additional hydrophobic groups were installed on the rigid spiropyrrolidine ring to mimic the side chain of Phe19 and Leu26. Initial compounds were designed with different hydrophobic groups with different stereochemistry and docked into the MDM2 binding cleft using the GOLD program [43, 44]. Docking studies showed that one such compound (MI-5, Fig. 3) closely mimics p53 in its interaction with MDM2. An efficient synthetic method featuring a [1,3]-dipolar cycloaddition reaction as the key step was developed for the synthesis of the designed compound. An FP-based binding assay confirmed that MI-5 binds to MDM2 with a K_i of 8.5 μM. Structure-based optimization of MI-5 ultimately yielded a new class of highly potent, small-molecule inhibitors, such as MI-219 (Fig. 3) [50, 51]. The most potent reported spirooxindole compounds have K_i values of <1 nM to MDM2. This class of compounds also displays exceedingly high specificity over other protein-protein interactions.

3 Representative Classes of Small-Molecule Inhibitors of the MDM2-p53 Interaction

Regardless of how initial lead compounds are discovered, extensive modifications are normally needed to obtain compounds with high affinities to MDM2, high specificity over other proteins, and good physiochemical and pharmacological properties. In this section, we summarize representative classes of potent, non-peptide, small-molecule inhibitors of the MDM2-p53 interaction from different research groups.

To the Nutlins, the first class of potent, non-peptide, small-molecule inhibitors of the MDM2-p53 interaction, extensive modifications have been carried out by scientists from Hoffmann-La Roche. One such compound, RG7112, binds to MDM2 with a K_d of 10.7 nM (Fig. 4) [52] and is the first MDM2 inhibitor to advance into Phase I clinical trials. Daiichi Sankyo has also designed potent MDM2 inhibitors starting from the core structure of Nutlins. The most potent compound **6**, (Fig. 4) disclosed in a patent has an IC_{50} of 1.2 nM [53].

Extensive modifications have been made on spirooxindoles by the Wang group at the University of Michigan [48, 49]. MI-147 is one of the most potent of this class of compounds, with a K_i of 0.6 nM in an FP-based competitive binding assay [51]. Additional modifications yielded new compounds with high affinities to MDM2 and improved pharmacokinetic properties [54]. One such compound in this class, MI-773, has completed IND-enabling studies and is entering clinical trials in 2012.

Starting from spirooxindoles, Hoffmann-La Roche has obtained several classes of potent MDM2 inhibitors through extensive chemical modifications. One class of compounds is structurally closely related to the spirooxindoles reported by the University of Michigan but these compounds differ in their stereochemistry [55, 56]. The most potent compounds disclosed in patents from Hoffmann-La Roche bind to MDM2 with IC_{50} values in the low nanomolar range.

Doemling's group from the University of Pittsburgh has reported a series of MDM2 inhibitors obtained through computational design and multicomponent reaction (MCR) chemistry [57]. One class of compounds contains a pyrazole or imidazole core linked with three hydrophobic groups: one indole ring and two phenyl rings. One of the most potent compounds YH265, (Fig. 4) has a K_i of 20 nM. Independently and simultaneously, Novartis disclosed a similar imidazole-indole family of compounds as inhibitors of MDM2 and MDMX in a patent [58]. The most potent compound has an IC_{50} of 15 nM for MDM2 in an FP binding assay and an IC_{50} of 1.32 μM for the related protein MDMX in a TR-FRET binding assay.

Novartis disclosed tetra-substituted heteroaryl compounds as MDM2 and/or MDMX inhibitors in a patent [59]. One of the most potent compounds in this class (**7**) has an IC_{50} value of 2 nM to MDM2. Interestingly, this compound also binds to MDMX with low micromolar binding affinity. Novartis also reported substituted isoquinolinones and quinazolinones as inhibitors of MDM2 and MDMX in a separate patent [60]. The most potent compound (**8**) has IC_{50} values of 0.8 nM and 2.1 μM for MDM2 and MDMX, respectively.

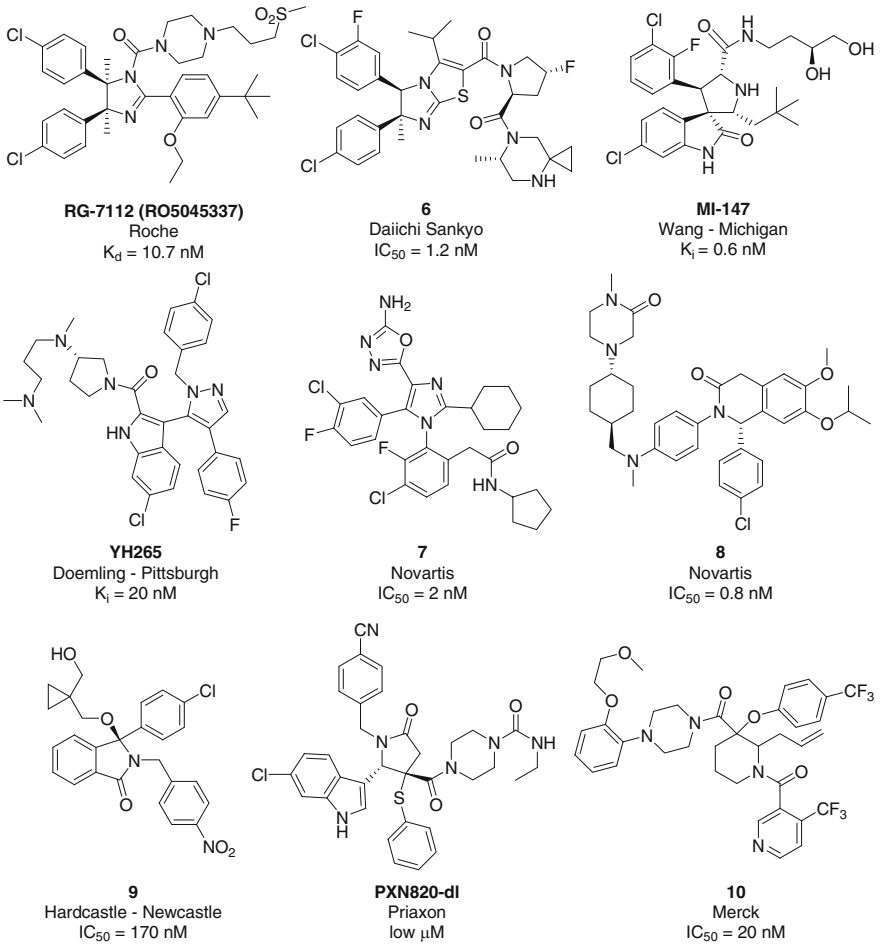

Fig. 4 Chemical structures and binding affinities of representative classes of MDM2 inhibitors

Researchers from Newcastle University reported a series of isoindolinone inhibitors for MDM2, the most potent of which **9**, (Fig. 4) has an IC_{50} value of 170 nM [61]. Priaxon AG Company reported a series of pyrrolidinone compounds as MDM2 inhibitors with IC_{50} values in the low micromolar range, exemplified by PXN820-dl in Fig. 4 [62]. A Merck patent reported highly substituted piperidines as MDM2 inhibitors. The most potent compound in this class, **10**, has an IC_{50} of 20 nM [63].

Since many of these MDM2 inhibitors have only been disclosed in patents, little is known about their cellular activity, pharmacological properties, and mechanism of action. Nevertheless, the successful discovery of these structurally diverse and potent small-molecule MDM2 inhibitors has clearly demonstrated that it is indeed possible to target the MDM2-p53 protein-protein interaction using non-peptide

small-molecule inhibitors. Although the binding affinities of these compounds have not been directly compared to each other under the same assay conditions, it is clear that the most potent, non-peptide, small-molecule MDM2 inhibitors discovered to date are >1,000-times more potent than wild-type p53 peptides.

4 Structural Basis of Binding of Small-Molecule Inhibitors to MDM2

A number of X-ray crystal structures of MDM2 complexed with potent small-molecule inhibitors of the MDM2-p53 interaction have been determined, providing structural insights for their high-affinity binding to MDM2. The first crystal structure of MDM2 complexed with a small molecule, Nutlin-2 [33], shows that Nutlin-2 mimics the interactions of the p53 peptide (Fig. 5a). One bromophenyl moiety in Nutlin-2 fills the Trp23 pocket, the other occupies the Leu26 pocket, and the ethyl ether side chain targets the Phe19 pocket. This crystal structure thus demonstrated that by mimicking the three key hydrophobic residues in p53, non-peptide, small-molecule inhibitors can achieve high affinities to MDM2. Interestingly, while Nutlin-2 mimics the three key hydrophobic interactions, it does not form a hydrogen bond like the one between the Trp23 indole NH group in p53 and the carbonyl group of Leu54 in MDM2.

A crystal structure of MDM2 complexed with a spirooxindole compound was determined (Fig. 5b). This compound is an analogue of MI-219, but differs in its stereochemistry [64]. Consistent with the initial design of this class of compounds, the oxindole inserts into the binding cavity occupied by Trp23 in p53 and nicely mimics both the hydrophobic and hydrogen-bonding interactions of the Trp23 indole. Interestingly, the halogen-substituted phenyl group in the MI-219 analogue binds to the hydrophobic pocket occupied by Leu26 in p53 and the aliphatic hydrophobic group mimics the hydrophobic side chain of Phe19. Additionally, π–π stacking takes place between the halogen-substituted phenyl group and the aromatic His96 in MDM2 and the compound also forms a hydrogen bond with His96, neither of which are observed in interactions between p53 peptides and MDM2.

The crystal structure of a benzodiazepine compound in complex with MDM2 shows that this compound uses three phenyl groups to mimic the three hydrophobic residues in p53 (Fig. 5c) [34]. No hydrogen bonding is observed between the inhibitor and MDM2. Similarly, the crystal structure of WK23, a potent inhibitor from Novartis, reveals that this compound also employs three aromatic groups to mimic the three hydrophobic residues in p53 (Fig. 5d) [64]. A hydrogen bond is formed between the indole NH group of Trp23 and the carbonyl group of Leu54 in MDM2.

Taken together, these high-resolution crystal structures have established a common interaction motif whereby all of the potent inhibitors mimic the three key residues (Phe19, Trp23 and Leu26) for hydrophobic interactions with MDM2. Interestingly, some classes of compounds such as the Nutlins and benzodiazepines

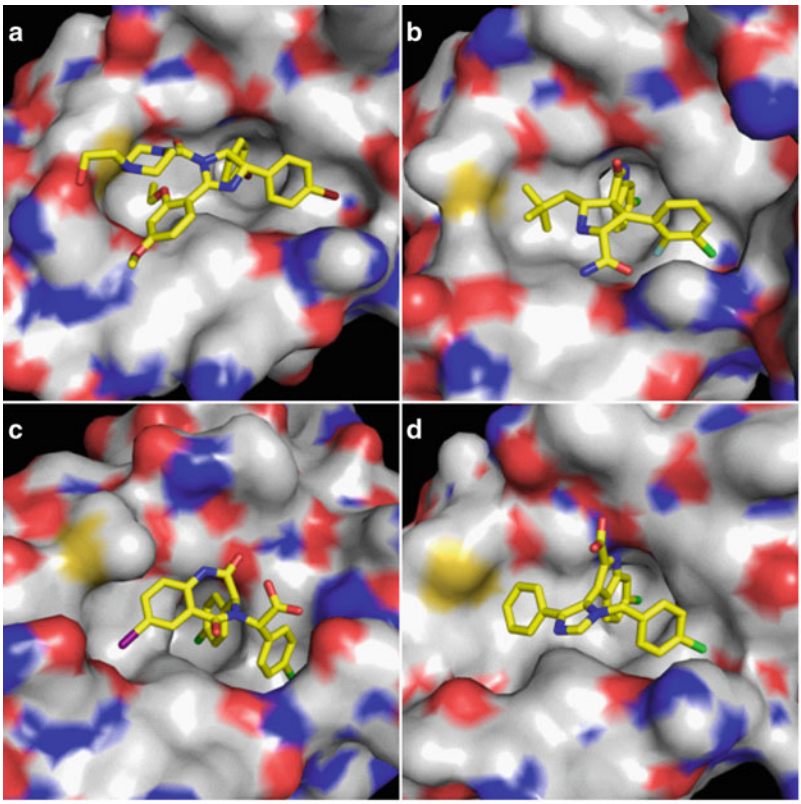

Fig. 5 Crystal structures of MDM2 complexed with several classes of inhibitors. (**a**) Nutlin-2; (**b**) MI-219 analogue; (**c**) benzodiazepine; (**d**) WK23 (PDB codes 1RV1, 3LBL, 1T4E and 3LBK, respectively)

lack the hydrogen bond formed between the indole NH group of Trp23 in p53 and the carbonyl group of Leu54 in MDM2.

5 Biological Aspects of MDM2 Inhibition

5.1 Molecular Mechanism of p53 Activation by MDM2 Inhibitors

Potent and specific MDM2 inhibitors such as Nutlin-3 [33] and MI-219 [50, 51] have provided an opportunity to examine the details of their cellular mechanism of p53 activation. Consistent with in vitro biochemical binding assays, potent MDM2 inhibitors are capable of blocking the MDM2-p53 protein-protein interaction in cells. They induce accumulation of p53 protein but do not increase the transcription

of the p53 gene in either tumor or normal cells with wild-type p53. Instead, they induce transcription of the p53-targeted genes for p21 and MDM2, increasing their protein levels. As compared to conventional genotoxic anticancer agents and radiation, activation of p53 by MDM2 inhibitors does not require phosphorylation of p53. Furthermore, it has been demonstrated that certain proteins, such as the pro-apoptotic Puma, are selectively induced in tumor cells but not in normal cells [50]. Therefore, although p53 is activated by MDM2 inhibitors in both normal and tumor cells with wild-type p53, the genes transcribed by p53 activation in these two settings are not identical, which may translate into different cell fates and have a significant implication for the potential applications of MDM2 inhibitors as new anticancer agents.

5.2 Activity and Selectivity of Small-Molecule MDM2 Inhibitors in Tumor and Normal Cells

Since MDM2 inhibitors activate p53 in both tumor and normal cells with wild-type p53, they can elicit a wide variety of cellular responses attributable to p53 activation. A number of investigations using Nutlin-3, MI-219, and other highly potent MDM2 inhibitors have demonstrated that MDM2 inhibitors result in both common and different cellular responses in normal and tumor cells [33, 50]. In normal cells, activation of p53 by MDM2 inhibitors induces cell cycle arrest but not cell death. In some but not all tumor cells, activation of p53 by MDM2 inhibitors induces both cell cycle arrest and cell death [33, 50, 65]. For example, Nutlin-3 and MI-219 induce robust cell death in SJSA-1 osteosarcoma and RS4;11 acute lymphoblastic leukemia (ALL) cell lines. In contrast, Nutlin-3 and MI-219 have minimal effect on cell death induction in HCT-116 colon, 22Rv1 prostate, and MCF-7 breast cancer cell lines, highlighting that p53 wild-type status in cancer cells is necessary but not sufficient for induction of cell death by MDM2 inhibitors [65]. Both cell-death induction and cell cycle arrest in tumor cells depend upon p53, since knockdown or knockout of p53 abrogates these cellular effects by Nutlin-3 and MI-219 [50, 66]. In addition to human cancer cell lines, cell death induction by MI-63 and Nutlin-3 were extensively evaluated using purified primary human chronic lymphocytic leukemia (CLL) cells [67] and acute myelogenous leukemia (AML) cells [68] from patients. Interestingly, while these MDM2 inhibitors effectively induce cell death in essentially all CLL cells with wild-type p53 status, they selectively kill some but not all AML cells harboring wild-type p53. Detailed genetic analysis revealed that 80% of AML cells with wild-type p53 and FLT-3 internal tandem duplication (FLT-3/ITD) mutation are highly sensitive to apoptosis via MDM2 inhibitors [68].

5.3 Antitumor Activity of MDM2 Inhibitors in Animal Models

A number of potent MDM2 inhibitors have been evaluated in animal models of human cancer for their antitumor activity. In pharmacodynamic experiments, a single oral dose of Nutlin-3 or MI-219 is capable of activating p53 in xenograft tumor tissues of human cancer cell lines with wild-type p53, as revealed by accumulation of p53 and upregulation of p21 [50, 65]. Both Nutlin-3 [33, 65, 69] and MI-219 [50] show strong antitumor activity in some but not all xenograft models of human cancer with wild-type p53. For example, while Nutlin-3 and MI-219 are capable of completely inhibiting tumor growth against SJSA-1 osteosarcoma xenografts, even causing some partial tumor regression, both compounds show minimal antitumor activity against HCT-116 colon cancer xenografts. These in vivo data are consistent with the in vitro robust cell-death induction by Nutlin-3 and MI-219 in the SJSA-1 cell line but not in HCT-116 cells. Immunohistochemical (IHC) analysis showed that MI-219 induced both cell cycle arrest and apoptosis in SJSA-1 xenograft tumor tissues. Significantly, the antitumor activity of these potent MDM2 inhibitors is achieved at dose schedules which cause no visible signs of toxicity in the animals, as assessed by necropsy studies and body weight loss [33, 50, 65, 69].

Although Nutlin-3 and MI-219 failed to achieve complete tumor regression in any of the tumor models tested, subsequent studies have shown that potent MDM2 inhibitors with optimized pharmacological properties can do so. For example, RG7112 achieved either a maintained complete response (MCR) or a complete response (CR) for a medulloblastoma and an alveolar rhabdomyosarcoma, respectively, and partial responses (PR) for a Wilms tumor, rhabdoid tumor, and Ewing tumor xenograft [70]. RG7112 was also highly effective against ALL xenografts: among 13 ALL xenografts, there were 11 CRs, one MCR, and one partial response [70]. In vivo studies on two compounds from the spirooxindole class also achieved rapid and complete tumor regression in SJSA-1 osterosarcoma and ALL RS4;11 xenograft models when dosed daily for 14 days [54]. In fact, a single dose of one such compound is capable of achieving complete tumor regression in the SJSA-1 xenograft model. Taken together, these preclinical data have provided compelling evidence that potent and highly optimized MDM2 inhibitors can achieve impressive antitumor activity in animal models of human cancers.

5.4 Potential Toxicity of MDM2 Inhibitors to Normal Tissues

The potential toxicity of p53 activation by an MDM2 inhibitor in normal tissues is of paramount importance for therapeutic development. Certain tissues such as bone marrow, small-intestine crypts, and thymus are highly susceptible to p53-induced apoptosis [71, 72]. Furthermore, activation of p53 by a genetic approach in the absence of *MDM2* results in severe pathological damage to p53-sensitive mouse

tissues and death of all animals within days [73], raising the possibility that MDM2 inhibitors could be highly toxic to some normal tissues through activation of p53.

To address this issue, a detailed examination of the toxicity of MI-219 in normal tissues was conducted [50]. While both γ-radiation and irinotecan chemotherapy induce robust apoptosis in small-intestine crypts and thymus, MI-219, in either single or repeated doses, did not cause apoptosis or damage in either radio-sensitive or radio-resistant normal mouse tissues. One reason for the lack of toxicity of MDM2 inhibitors in normal mouse tissues is that activation of p53 in the presence of MDM2 is transient; when the concentrations of MDM2 inhibitors decrease from tissues due to pharmacokinetics, MDM2 protein can bind to p53 and rapidly reduce the levels of p53. Furthermore, in the presence of MDM2, p53 action is always under the control of MDM2, in contrast to activation of p53 in the absence of MDM2 in genetic models. While the data in mice provide encouraging evidence for the therapeutic index of MDM2 inhibitors, the ultimate answer will need to come from human clinical trials.

5.5 *MDMX Is a Modulator of the Activity of Selective MDM2 Inhibitors*

MDMX is a homolog of MDM2. Similarly to MDM2, MDMX also binds directly to p53 and inhibits its transcriptional activity. But unlike MDM2, MDMX does not induce p53 degradation [74].

Crystal structures of MDM2 and MDMX reveal that these two proteins have very similar p53 binding pockets. However, most of the reported MDM2 inhibitors are highly selective for MDM2 over MDMX. For example, although Nutlin-3 still binds to MDMX, its affinity is >100-fold less than that toward MDM2. MI-219 binds to MDM2 with a low nanomolar affinity, but is >1,000-fold weaker to MDMX [50]. When MDMX is overexpressed in tumor cells, selective MDM2 inhibitors may not be able to fully activate p53. Indeed, ectopic expression of MDMX and/or its downregulation by RNAi show that MDMX attenuates the p53 activation by selective MDM2 inhibitors and reduces the cellular activity of MDM2 inhibitors [50, 66, 75, 76]. Interestingly, in some cancer cell lines, MDM2 inhibitors can induce MDMX degradation, presumably mediated though p53-dependent up-regulation of MDM2 [50, 66, 76], which is known to ubiquitinate and degrade MDMX during DNA damage [77]. Such cancer cell lines are more susceptible to Nutlin-3 than those in which Nutlin-3 fails to induce MDMX degradation. Though it is unclear why MDM2 inhibitors can induce MDMX in some cancer cell lines but fail to do so in other cancer cell lines, MDMX has nevertheless emerged as an important modulator for the activity of selective MDM2 inhibitors, which provides an impetus for the design of dual small molecule inhibitors targeting both MDM2 and MDMX.

5.6 p53-Independent Effects of MDM2 Inhibitors

In addition to p53, MDM2 also binds to other proteins such as p73, E2F-1, HIF-1α, and Numb [78–81] using the same binding site. Therefore, small-molecule MDM2 inhibitors that bind to the p53-binding site in MDM2 should also interfere with the binding of MDM2 to these proteins. Using in vitro cell line models, it has been shown that Nutlin-3 disrupts the interaction of MDM2 with p73 [78], E2F-1 [79] and HIF-1α [80]. However, higher concentrations of Nutlin-3 (for a p73-dependent effect) or a DNA damage signal (for an E2F-1-dependent effect) were required for p53-independent effects to be observed. Further studies are needed to determine the contributions of both p53-dependent and p53-indepdenent effects for the overall cellular activity of MDM2 inhibitors.

5.7 MDM2 Inhibitors as Potential Anti-angiogenic Agents

In addition to having a direct effect on tumor cells, MDM2 inhibitors may inhibit angiogenesis through activation of p53 in endothelial cells [82, 83]. Activation of p53 can upregulate several anti-angiogenic factors, including thrombospondin-1 (TSP-1) and brain-specific angiogenesis inhibitor 1 (BAI1), and down-regulate several pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), basic fibroblast growth factor binding protein (bFGF-BP), and cyclooxygenase-2 (COX-2) [1]. The in vitro and in vivo anti-angiogenic activity of MDM2 inhibitors has been demonstrated using Nutlin-3 [82]. By inhibiting angiogenesis, MDM2 inhibitors may have an application for the treatment of tumors lacking functional p53.

5.8 Predictors of Response to MDM2 Inhibitors

In tumor cells harboring wild-type p53, activation of p53 by MDM2 inhibitors can induce cell cycle arrest and/or apoptosis. Extensive studies have shown that while MDM2 inhibitors induce cell cycle arrest in all tumor cells with wild-type p53, they induce apoptosis/cell-death only in some tumor cells harboring wild-type p53 [33, 50, 66, 84]. Hence, p53 wild-type status is necessary but not sufficient for apoptosis induction by MDM2 inhibitors. Therefore, it is critical to identify biomarkers for predicting the antitumor activity of MDM2 inhibitors.

The SJSA-1 osterosarcoma cell line has an amplified *MDM2* gene and very high levels of MDM2 protein. Nutlin-3 and MI-219 are very effective in inhibition of cell growth and induction of apoptosis in the SJSA-1 cell line in vitro and both compounds achieve strong antitumor activity against SJSA-1 xenograft tumors in mice. Therefore, tumors with MDM2 amplification may be highly sensitive to

MDM2 inhibitors. MDM2 amplification occurs in approximately 7% of human tumors. Different tumor types have widely varying degrees of MDM2 amplification, such as liposarcoma (50–90%), osteosarcomas (16%), esophageal carcinomas (13%), and colon cancer (9%). Furthermore, MDM2 amplification and p53 mutation are essentially mutually exclusive [22]. Therefore, MDM2 amplification could be a very useful biomarker for predicting the clinical response of an MDM2 inhibitor.

Studies using AML blasts from 109 patients have shown that all AML cases with mutated p53 were resistant to apoptosis induction by MI-219 [68]. Approximately 30% of AML cases with unmutated p53 showed primary resistance to MI-219. Analysis of potential mechanisms associated with MI-219 resistance in AML blasts with wild-type p53 uncovered a number of distinct molecular defects, including low or absent p53 protein induction after MDM2 inhibitor treatment. For a separate subset of resistant blasts, robust p53 protein induction after MI-219 treatment was observed, indicative of defective p53 protein function or defects in the apoptotic p53 network. Interestingly, 80% of AML blasts with mutated Flt3 status (Flt3-ITD) and wild-type p53 are highly sensitive to apoptosis induction by MI-219. Therefore, MDM2 inhibitors could be particularly useful for the treatment of AML with mutated Flt3 status, which constitutes a clinically high-risk group of AML. Since a number of MDM2 inhibitors have now entered human clinical trials, it is expected that analysis of clinical efficacy data and human tumor samples will shed new light on potential biomarkers for the anti-tumor activity of MDM2 inhibitors.

5.9 MDM2 Inhibitors in Combination with Other Anticancer Drugs

A number of studies have demonstrated that MDM2 inhibitors may be used not just as single agents but also in combination with other anticancer drugs to achieve better antitumor activity than single agents. For example, ex vivo experiments using patient tumor samples have shown that Nutlin-3 synergizes with doxorubicin, chlorambucil, and fludarabine in B-CLL [85–87]; with doxorubicin and cytosine arabinoside in AML [88, 89]; and with doxorubicin in Hodgkin and Reed-Sternberg (HRS) cells [90]. Significantly, in these experiments Nutlin-3 as a single agent or in combination was non-toxic towards normal hematopoietic cells. Combination of Nutlin-3 with velcade, a proteasome inhibitor approved for the treatment of multiple myeloma, showed a synergistic activity in multiple myeloma cells harboring wild-type p53 [91]. MI-147, a potent member of the spirooxindole class of MDM2 inhibitor, enhanced the antitumor activity of irinotecan against SJSA-1 xenografts in mice [51]. These studies have provided evidence that MDM2 inhibitors can be developed in combination with other types of anticancer drugs.

5.10 Acquired Resistance to MDM2 Inhibitors

Since p53 activation is critical for the antitumor activity of MDM2 inhibitors, persistent exposure to MDM2 inhibitors may select for tumors that are defective in p53 function. Indeed, a recent study demonstrated that Nutlin treatment in SJSA-1 cells leads to the acquisition of somatic mutations in p53 and selects for p53-mutated cells. Such cells are unable to undergo cell cycle arrest or apoptosis when treated with Nutlins [92]. Since tumor cells can acquire resistance to MDM2 inhibitors through p53 mutation, the combination of MDM2 antagonists with agents that can target p53-mutated cells might potentially limit the impact of selecting for p53-mutated tumors through treatment with MDM2 inhibitors.

5.11 MDM2 Inhibitors in Clinical Development

The first MDM2 inhibitor that entered clinical development is RG7112 (RO5045337, Fig. 4) from Hoffmann-La Roche (clinicaltrials.gov identifiers: NCT01164033, NCT01143740, NCT00623870, and NCT00559533). Four Phase I clinical trials have been conducted to date in patients with advanced solid tumors, hematologic neoplasms, or liposarcomas prior to debulking surgery. Preliminary clinical data indicated that RG7112 appears to be well tolerated in patients and shows initial evidence of clinical activity and a mechanism of action consistent with targeting of the MDM2-p53 interaction [93–95].

Another MDM2 inhibitor from Hoffmann-La Roche, RO5503781, whose structure has not been disclosed, entered into Phase I clinical trials at the end of 2011 (clinicaltrials.gov identifier: NCT01462175). A spirooxindole class of MDM2 inhibitor discovered at the University of Michigan has completed IND-enabling studies by Sanofi and Phase I clinical trials are expected to begin in 2012.

6 Conclusions

Because of the powerful tumor suppressor function of p53, reactivation of p53 has been long sought as a potentially novel cancer therapeutic strategy. In tumors harboring wild-type p53, MDM2 is the primary cellular inhibitor of p53 activity. Since MDM2 was found to inhibit p53 through a direct protein-protein interaction, blocking this interaction using small molecules was pursued in several academic and industrial pharmaceutical laboratories in the last decade.

High-resolution crystal structures of MDM2 complexed with p53 peptides defined the interaction in detail, revealing that the MDM2-p53 interface has a well-defined, relatively compact binding pocket in MDM2, which suggested that for this particular

protein-protein interaction, it may be feasible to design potent, non-peptide, druglike small-molecule inhibitors to block the p53-MDM2 interaction.

A variety of approaches have been employed to identify initial non-peptide lead compounds, including experimental screening of small and large chemical libraries, computational structure-based screening of large chemical libraries, followed by experimental testing and computational structure-based *de novo* design. Extensive chemical modifications have been performed on several classes of compounds, including the Nutlins from Roche and the spirooxindole class from the Wang laboratory at the University of Michigan. These efforts have led to the discovery of several classes of highly potent and specific small-molecule inhibitors of the MDM2-p53 interaction, with suitable physiochemical and pharmacological properties for clinical development. To date, at least three compounds have been advanced into early clinical development, including two compounds from Roche and one compound from the University of Michigan. The first clinical compound, RG7112, from Roche has demonstrated good tolerability and initial evidence of clinical activity in patients with advanced solid tumors and leukemia. Hence, these MDM2 inhibitors may prove to be a class of completely novel anticancer therapy for the treatment of many different types of human cancer.

Acknowledgements Funding from the National Cancer Institute/National Institutes of Health, the Prostate Cancer Foundation, the Leukemia and Lymphoma Society, Ascenta Therapeutics and Sanofi is greatly appreciated.

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http://www.springer.com/978-3-642-28964-4

Protein-Protein Interactions
Wendt, M.D. (Ed.)
2012, XVIII, 262 p., Hardcover
ISBN: 978-3-642-28964-4