

Small Molecule Antagonists of the MDM2 Oncoprotein as Anticancer Agents

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Abstract: In this early phase of the new era of molecularly targeted patient friendly cancer chemotherapy, there is a need for novel viable anticancer molecular targets. The MDM2 oncoprotein has been validated as a potential target for cancer drug development. MDM2 amplification and/or overexpression occur in a wide variety of human cancers, several of which can be treated experimentally with MDM2 antagonists. MDM2 interacts primarily with the p53 tumor suppressor protein in an autoregulatory negative feedback loop to attenuate p53's cell cycle arrest and apoptosis functions. Inhibition of the p53-MDM2 interaction has been shown to cause selective cancer cell death, as well as sensitize cancer cells to chemotherapy or radiation effects. Consequently, this interaction has been the main focus of anticancer drug discovery targeted to MDM2. The promotion of the proteasomal degradation of the p53 protein by MDM2 is central to its repression of the tumor suppressor functions of p53, and many proteins impinge upon this activity, either enhancing or inhibiting it. MDM2 also has oncogenic activity independent of its interaction with p53, but this has so far not been explored for drug discovery. Among the approaches for targeting MDM2 for cancer therapy, small molecule antagonists have recently featured as effective anticancer agents in experimental models, although the repertoire is currently limited and none has yet entered human clinical trials. Small molecules that have been reported to disrupt the p53-MDM2 binding, thereby enhancing p53 activity to elicit anticancer effects include the following: synthetic chalcones, norbornane derivatives, *cis*-imidazoline derivatives (Nutlins), a pyrazolidinedione sulfonamide and 1,4-benzodiazepine-2,5-diones, as well as tryptophan derivatives. In addition to compounds disrupting p53pMDM2 binding, three compounds have been discovered that are effective in inhibiting the E3 ligase activity of MDM2 towards p53, and should serve as leads for drug discovery targeting this aspect of the p53-MDM2 interaction as well. These compounds were discovered from library screening and/or structure-based rational drug design strategies.

Keywords: MDM2, p53, small molecules, anticancer, drug design, drug discovery, protein-protein interaction.

INTRODUCTION

MDM2 (also frequently referred to as HDM2 in human) is a zinc finger oncoprotein [1-4] that has been well characterized as the principal negative regulator of the p53 tumor suppressor protein [5]. p53 is a transcription factor that is pivotal to cellular responses to genotoxic and other stress [6-8]. In this capacity, wild type p53 function is critical for maintaining the genomic integrity of cells. The "genome guardian" or tumor suppressor functions of p53 stem from transcription-dependent or -independent induction of apoptosis (programmed cell death), cell cycle arrest, differentiation and/or senescence, as well as its involvement in DNA repair [7, 9-19]. Under physiological conditions, p53 appears to be inactive, and its levels are kept very low, mainly due to destabilization by MDM2 [6, 20-22]. This allows for normal balance in cell growth, proliferation and survival. On the other hand, under genotoxic (DNA damage) or other stress conditions such as oncogenic activation, oxidation, hypoxia, ribonucleotide depletion or mitotic spindle damage, p53 becomes activated and stabilized, resulting in cell cycle arrest or apoptosis [7, 8, 23].

Loss of function *p53* (*TP53*) gene mutations occur in about half of human cancers [24-26], resulting in more aggressive and drug resistant tumor phenotypes [27, 28]. In the other half of human cancers, which harbor a wild type p53, its tumor suppressor functions can still be compromised by the overexpression or deregulation of MDM2 [6, 29], also conferring tumor aggressiveness and drug resistance. The inhibition of MDM2 function in these cancers is, therefore, viewed as an attractive means of triggering or enhancing cancer cell death by promoting p53-induced cell cycle arrest and/or apoptosis [30-33]. This overview focuses on the targeting of the MDM2 with small molecules for novel approaches to selective cancer therapy, highlighting classes of compounds that have been reported to inhibit p53-MDM2 interactions. Other known interactions and activities of MDM2 that may also be harnessed for targeted cancer therapy are considered as well. Anticancer strategies based on the use of MDM2 inhibitory polypeptides [31, 34-38], MDM2 antibodies [39], or the knock-down of MDM2 gene expression (i.e. antisense oligonucleotides, siRNA) (for reviews see refs. [30, 40-42]), will not be covered.

ONCOGENIC ACTIVITIES OF MDM2

The oncogenic properties of the MDM2 protein result from its functions as a major p53 repressor, as well as p53-

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independent effects (see reviews by Rayburn *et al.* in this issue, ref. [43] and Zhang and Zhang in this issue, ref. [44]).

Repression of p53 by MDM2

The relationship between p53 and MDM2 is an interesting one. p53 transcriptionally induces the expression of MDM2 as a means of keeping itself in check, in an autoregulatory negative feedback loop [20, 45-48]. This has led to MDM2 being dubbed p53's 'big brother' [49]. The p53 down-regulatory activity of MDM2 is essential to normal cell function. Thus, the deletion of the *MDM2* gene in knockout mice causes death at the embryonic stage unless the *TP53* gene (the gene that encodes the p53 protein) is simultaneously deleted [50, 51]. The ectopic overexpression of MDM2 in cells in culture, has been shown to cause an inhibition of both cell cycle arrest and apoptosis [52].

The negative regulation of p53 by MDM2, which is central to our current view of MDM2 as an oncogene, occurs at the post-translational level in at least three ways: (i) binding and physical occlusion, (ii) induction of proteasomal proteolysis and (iii) exportation of p53 from the nucleus. MDM2 binds p53 at the N-terminal transactivation domain at its N-terminal domain [35, 53], resulting in physical interference with p53's transcriptional interactions and abrogation of transcriptional activity [4, 54-56]. After binding to p53, MDM2 acts as an E3 ubiquitin ligase [57] using its C-terminal RING finger domain to conjugate ubiquitin to the C-terminal domain of p53, and by that, target p53 for proteasomal degradation [58-61]. It has also been reported that MDM2 can promote p53 proteasomal degradation without direct binding [62]. MDM2 also uses its RING finger domain to tag itself for proteasomal degradation as well [63]. In addition to the E3 ligase activity, the acidic domain of MDM2 is required for the proteasomal degradation of both p53 and MDM2 itself [63]. It has also been observed that, in addition to ubiquitination, MDM2 also NEDDylates p53 as an additional means of destabilizing it [64]. It should be mentioned that MDM2-independent p53 degradation mechanisms also exist. For example, jun-N terminal kinase (JNK)-dependent proteasomal degradation [65, 66] and non-proteasomal calpain-mediated degradation have been reported [67-70]. However, these alternative degradation pathways do not appear to play a significant role in normal p53 regulation.

Positive and Negative Modulation of the Repression of p53 by MDM2

Several factors modulate the destabilization of p53 by MDM2 in positive or negative ways. For example, the oncogenic kinase Akt, phosphorylates MDM2 to stabilize it and promote its anti-p53 function [71-73], whereas c-Abl tyrosine kinase phosphorylates MDM2 to inhibit its ubiquitination of p53 [74, 75]. Sumoylation of MDM2, which is carried out by the SUMO-1 conjugating enzyme Ubc9, prevents MDM2 self-ubiquitination while facilitating p53 ubiquitination by MDM2 [76, 77]. Acetylation of MDM2 by proteins like CREB-binding protein (CBP) inhibits its p53 regulatory function [78]. Further, MDM2 can bind to several other proteins that affect its ability to induce p53 degradation (reviewed in [61, 79]). Some of these

proteins inhibit its p53 repressive effects such as ARF, which sequesters MDM2 to the nucleolus away from p53, and directly inhibits p53 ubiquitination by MDM2 [80-83]. pRb and p300, [48, 79, 84] as well as the ribosomal proteins L5, L11 and L23 [85-90] and the tumor suppressor Merlin (a product of the *NF2* gene [91, 92]) also bind to and inhibit MDM2 ubiquitin ligase activity towards p53. It has been reported that Seladin 1, a key mediator of Ras induced senescence, binds to p53 at the amino terminal region that binds to MDM2, thus displacing MDM2 from p53 and enhancing p53 accumulation [93]. Further, the FHIT tumor suppressor protein has been shown to interact with MDM2 to inactivate it and promote p53 stability by a mechanism that appears to involve an effect on the phosphorylation status of MDM2 [94].

Many proteins have also been shown to bind to MDM2 and enhance its capacity to degrade p53. They include TSG101, which stabilizes MDM2 by inhibiting its self ubiquitination [61, 79, 95], as well as Cul4A [96], PML [97], -arrestin 2 [98], and MDMX (MDM4), a closely related MDM2 homologue that shares several highly conserved regions [99], and is required to regulate the growth suppressor properties of p53, which binds to MDM2 and promote p53 degradation. Interestingly, cyclin G recruits MDM2 to protein phosphatase 2A (PP2A) for dephosphorylation and activation [100]. MDM2 also recruits the histone deacetylase HDAC1 and deacetylate p53 to enhance its ubiquitination by MDM2 for proteasomal degradation [101]. The release of p53 in the p53-MDM2 feedback loop is thought to occur in well-timed quanta until DNA damage is repaired or the cell death occurs [102].

A third mechanism of MDM2 regulation of p53 is nucleo-cytoplasmic export [103]. This export depletes p53 from the nucleus, thus abrogating its transcriptional activity. It was originally thought that the nuclear export of p53 was required for the proteasomal degradation of p53 [104, 89], but recent data indicate otherwise, showing that p53 is degraded both inside and outside the nucleus [105]. The apparent controversy now appears cleared by the demonstration that when p53 is only mono-ubiquitinated under condition of low MDM2 levels, it is exported to the cytoplasm for degradation, but when MDM2 levels are high, p53 is polyubiquitinated in the nucleus and is degraded there [106]. Several reviews provide excellent discussions of MDM2 as a p53 regulator, as well as its interaction with various other cellular proteins [29, 48, 107-110].

p53-Independent Oncogenicity

In addition to the well-known downregulation of p53 by MDM2 as major contributor to MDM2's oncogenic effects, several MDM2 interactions with other cellular proteins have also been identified as a basis for the oncogenic activity of MDM2 that are independent of p53 [111].

The following are several examples of these p53-independent interactions of MDM2:

- 1) *MDM2-p73 interaction:* p73 is a close homologue of p53 that also triggers apoptosis, and can activate p53 target genes [112, 113]. In fact, the manner in which p73 functions similar to p53 has led to it being ascribed the title of "assistant" guardian of the

genome [114]. MDM2 physically associates with p73 and interferes with its functions. Unlike the case with p53, MDM2 binding to p73 does not cause its degradation, but only the blockade of its transcriptional activity by displacing it from its complex with p300/CBP necessary for p73 transactivational activity. The MDM2-p73 interaction is particularly interesting in light of the evidence that p73 is a determinant of chemosensitivity of tumors [115, 116]. It also appears that in cancers where p53 is mutated, the response to chemotherapy is mediated, at least in part, by p73 activation [114].

- 2) *MDM2-TIP60 interaction:* MDM2, through its E3 ligase activity, also targets the histone acetyltransferase (HAT) protein TIP60 (which is involved in induction of apoptosis and DNA repair following DNA damage [117]) for proteasomal degradation [118].
- 3) *MDM2-p21 interaction:* An interesting recent discovery is the finding that MDM2 also interacts with p21WAF1 and causes the degradation [39] of this cyclin-dependent kinase inhibitor, a key p53 transcriptional target protein that causes cell cycle arrest [119-122]. The p53-independence of this interaction was demonstrated by the use of MDM2 antisense or siRNA, which caused an elevation in p21 protein levels in the p53 null PC3 cell line. MDM2 causes p21 degradation, independent of its E3 ligase activity, but rather through recruitment of p21 to the proteasomal C8-subunit. The p21 binding region on MDM2 has been mapped to amino acid residues 180-298 of MDM2 [123]. This activity of MDM2 abrogates the cell cycle arrest effects of p21.
- 4) *MDM2-Numb interaction:* MDM2 binds and ubiquitinates Numb (an antagonist of Notch signaling, which promotes differentiation [124]), and induces its degradation [125].
- 5) *HIF-1 induction by MDM2:* The expression of the hypoxia-inducible factor 1 (HIF-1), a transcription factor that promotes tumor angiogenesis and progression [126], is positively modulated by MDM2 [127]. The induction of HIF-1 and MDM2 can occur in a p53 null context (p53^{-/-} HCT116), showing a p53-independent phenomenon. Hypoxic conditions that trigger HIF-1 overexpression are also known to upregulate MDM2 resulting in enhanced tumor metastatic capacity [128].

MDM2 in Human Cancer

The *Mdm2* gene was first cloned as an amplified gene on a murine double-minute chromosome [2] and was subsequently found to be amplified in a portion of human sarcomas [3] and brain tumors [129, 130]. The ability of MDM2 to block the cell cycle arrest and apoptotic effects of p53 implies that it can promote cell proliferation and survival, both of which are hallmarks of cancer [131]. This has been demonstrated in cell culture [2, 52].

The overexpression of MDM2 has been observed in a wide variety of human cancers, notably in sarcomas. The overexpression of MDM2 has also been shown in esophageal

carcinomas, brain tumors, breast, ovarian, cervical, lung, colon, prostate, bronchial, nasopharyngeal, testicular and urothelial cancers, and in neuroblastomas and pediatric solid tumors, among others [2, 30, 132]. Moreover, MDM2 overexpression generally portends a poor prognosis [133-135], particularly when it occurs together with mutant p53 [136]. Overexpression of MDM2 may result from one or more of three mechanisms: (i) gene amplification [132, 137], (ii) increased transcription [138] and (iii) enhanced translation [138].

The majority of the cancers overexpressing MDM2 also harbor wild type p53. The overexpression of MDM2 in these cancers might therefore have contributed to the transformation and their malignancy by repressing the tumor suppressor functions of p53. p53-independent MDM2 overexpression has also been observed. For example, the angiogenic growth factor, basic fibroblast growth factor (bFGF) has been shown to induce MDM2 protein overexpression independent of p53, to prolong cell survival and resistance to the anticancer drug cisplatin [139]. MDM2 is also upregulated in a p53-independent manner by tumor hypoxia, along with HIF-1 upregulation [128]. The involvement of MDM2 in human cancer has also been shown by the recent finding that a single nucleotide polymorphism, SNP309, in the MDM2 promoter results in a higher affinity for the transcriptional activator Sp1, leading to MDM2 overexpression, and is associated with accelerated tumor formation in both hereditary and sporadic cancers [140].

The current pursuit of small molecule MDM2 antagonists for cancer therapy is based on the rationale that relieving p53 repression by MDM2 will result in enhanced p53 activity in cancers with wild-type p53 [52]. Many anticancer agents exert their tumor growth inhibitory effects through induction of apoptosis in the context of functional p53 [141, 142]. The consequence of inhibiting MDM2 will be an increase in p53 levels and an increase in the expression of downstream p53 effectors leading to cell cycle arrest and/or apoptosis [143].

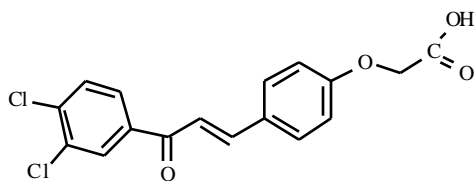
It has been amply demonstrated by the use of p53-derived peptides [34, 35], antisense approaches [30, 40-42, 144, 145], and small molecule inhibitors [31, 32, 146], that the p53-MDM2 interaction is a bonafide anticancer drug target. Moreover, it has been shown that inhibiting MDM2 does not cause major normal tissue toxicities [147], which augurs well for achieving selectivity in cancer therapy with MDM2 inhibitors. In addition to the potential use of MDM2 antagonists in cancer therapy in their own right, they also appear to be very attractive candidates for use as chemosensitization and/or radiation sensitization agents, since both cancer cell chemosensitivity and sensitivity to radiation treatment, have been shown to correlate with wild type p53 status [148, 149]. Experiments with antisense oligonucleotides show that this is feasible [122, 144].

SMALL MOLECULES ANTAGONISTS OF MDM2 FOR CANCER THERAPY

Targeting p53-MDM2 Binding with Small Molecules

In light of the recent successes achieved in the therapeutic exploitation of molecular targets in cancer cell proliferation and survival promoting signaling pathways, as exemplified by small molecule drugs Gleevec (imatinib, [150]), Iressa

(gefitinib, [151]) and Velcade (bortezomib, [152]), it is not surprising that serious efforts are underway to target p53 pathways with small molecules for cancer therapy. Small molecule drugs are attractive because of several advantages they have over large molecules like peptides, antibodies and polynucleotides (antisense oligonucleotides, ribozymes, siRNA etc.), such as better stability, delivery and bioavailability (especially oral bioavailability), as well as less regulatory control and lower production costs.



IC₅₀ = 49 μM (ELISA)
K_d = 90 μM (NMR)

Fig. (1). Representative chalcone carboxylic acid inhibitor.

The p53-MDM2 interaction is the best characterized of all MDM2 interactions and offers a logical target for exploitation in cancer therapy. Targeting p53-MDM2 interaction with small molecules is one of several approaches to activating p53 for cancer treatment [153, 154]. Protein-protein interactions are generally difficult to inhibit with small molecules due to factors like large and shallow interaction interfaces often lacking deep binding pockets, and difficult to cover with small molecules. This notwithstanding, progress is being made in this area [155, 156]. Fortunately, in the case of p53-MDM2 binding at the N-terminal of MDM2, the interface is relatively small, and has a well-defined deep basin-like cleft with well-defined hydrophobic pockets [53] that small molecules can bind with high affinity and effectively compete against p53 binding. Early successful studies with p53-derived peptides [34, 35, 143, 154] that showed that the MDM-p53 interaction could be effectively disrupted, opened the door for harnessing the interaction for therapeutic purposes. The assays that were developed through the peptide studies, i.e. ELISA and gel mobility shift assays [34, 35] paved the way for the search for inhibitors. Thus, several classes of compounds have been reported to exhibit high-affinity competitive binding to the p53 binding site on MDM2, and by that, to cause p53 accumulation and enhance cell cycle arrest, and/or apoptosis in cancer cells (for recent reviews, see refs. [31-33, 157-159]). The current increase in pace in discovery of small molecule

p53-MDM2 inhibitors can be attributed to more sophisticated structure-based drug design tools and better assays.

Screening Approaches to the Discovery of Small Molecule MDM2 Antagonists

In 2000, Stoll *et al.* reported the discovery of chalcones that bound to MDM2 at the p53 transactivation domain binding site using an ELISA assay that employed a p53 peptide [160]. These inhibitors were of low to moderate affinity, with IC₅₀ values in the 50-250 μM range and above. The binding of these chalcones on MDM2 was characterized by NMR spectroscopy as well, using a ¹⁵N-HSQC experiment. The binding data for the best compound are shown in Fig. (1).

The general low potency of these chalcones possibly reflects a failure of the groups to adequately occupy the hydrophobic pocket. A potential drawback of these α,β -unsaturated ketone derivatives is indiscriminate reaction with cellular nucleophiles including proteins and nucleic acids as Michael acceptors.

Through the screening of synthetic compound libraries, a group from the Roche company identified *cis*-imidazoline derivatives with suitable hydrophobic substituents well positioned to occupy the hydrophobic subpockets of the critical triad of amino acid residues that have been found to be necessary for p53 binding [146]. The compounds were named Nutlins (see Fig. (2)), and exhibited submicromolar IC₅₀ values (100-300 nM) in displacing p53 from its complex with MDM2. The investigators also demonstrated the potent, MDM2-inhibitory dependent anticancer effects of the Nutlins both *in vitro* in cell culture and *in vivo* in animal xenograft models [146]. Their study was the first real proof-of-principle that sole targeting of p53-MDM2 interaction is a viable target for targeted cancer therapy. One of the compounds, Nutlin 2 (Fig. (2)) was cocrystallized with MDM2 and the structure solved by X-ray diffraction.

The complex obtained showed that the compound bound with the imidazole ring as a scaffold projecting three hydrophobic groups into the three hydrophobic pockets like p53 does, as shown in Fig. (3), which depicts a superimposition of bound p53 peptide and Nutlin 2 in the MDM2 cleft. The Nutlin and p53 peptide bind analogously. The two vicinal 4- and 5-position *para*-bromophenyl substituents occupy the same positions as p53 residues Leu26 and Trp23, respectively, while the *ortho*-ethoxy

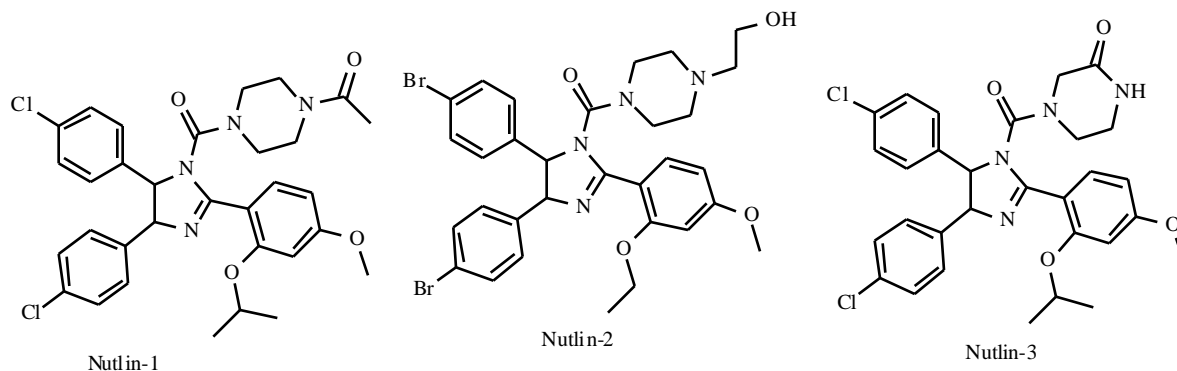


Fig. (2). Nutlins, *cis*-imidazoline derivative MDM2-p53 binding inhibitors and anticancer agents [146].

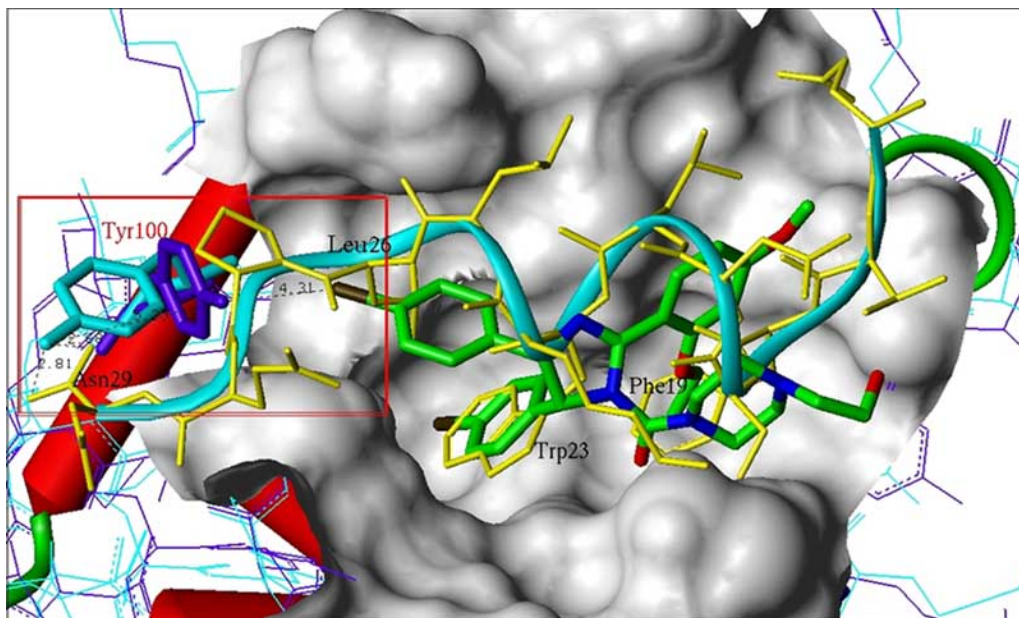
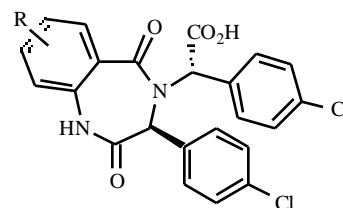


Fig. (3). Superimposition of Nutlin 2-MDM2 [146] and p53-MDM2 [53] cocrystal structures. The p53 peptide is colored yellow with a cyan ribbon showing the α -helix secondary structure. Nutlin 2 is colored according to atom type: C, green; N, blue; O, red; Br, brown. Hydrogens are not shown. The red box encloses a region showing the induced fitting of MDM2's Tyr100, purple in the Nutlin 2-MDM2 complex, and cyan in the p53-MDM2 complex. The black broken lines with numbers show interatomic distances in angstroms.

substituent on the 2-position phenyl substituent occupies the same pocket as p53 residue Phe19. Compared to the bound p53 peptide, however, there is an induced fit in the Nutlin-MDM2 complex involving MDM2 residue Tyr100 that is different from that in the p53-MDM2 complex. In the p53-MDM2 complex, this tyrosine residue of MDM2 hydrogen bonds with Asn29 of the p53 peptide, whereas in the Nutlin-MDM2 complex, this tyrosine move towards the cleft for 5.3 angstroms to bond with the *para*-bromo substituent on the imidazoline 4-position bromophenyl (see box in Fig. (3)). This shows a flexibility of this residue that may be taken advantage of to design different classes of inhibitors.

This implies that if one uses the p53-MDM2 complex structure for structure-based drug design, one can come up with entirely different ligands from those that will be obtained if the Nutlin-MDM2 complex structure was used for ligand design. The Nutlin-MDM2 structure has an induced fit at the receptor site that will be suitable for structure-based design of small molecule inhibitors, whereas the p53-MDM2 complex site will better serve peptide ligand discovery. This difference is important and calls for a study of the flexibility of residue Tyr100, which might be done by molecular dynamics simulations, to investigate its conformational preferences. This information would be useful for the design of different classes of compounds to increase the diversity of inhibitors for the p53-MDM2 target. The recent report by Zhong and Carlson [161] on the molecular dynamics simulation of the human p53-MDM2 complex is pertinent to this question. The authors used molecular dynamics simulations and computational alanine-scanning mutations to study the binding of a beta-peptide p53 mimic, and showed similarities between the mimic binding and Nutlins' binding to MDM2. They also reported the identification of an additional hydrophobic pocket in the interior of the MDM2 cleft.

Recently, a library of 22,000 1,4-benzodiazepine-2,5-diones was screened using the high throughput direct binding assay Thermofluor[®], for binding to the p53-binding domain of MDM2 [162]. The hits obtained were further shown to bind to MDM2 in the p53-binding pocket using a recently described fluorescence polarization (FP) peptide displacement assay [163]. The series was optimized for potency to obtain sub-micromolar antagonists of the p53-MDM2 interaction. The most potent antagonists (Fig. (4a)) possessed two chlorophenyl substituents that occupy two of the three hydrophobic pockets of the MDM2 cleft, while an iodophenyl or chlorophenyl group (iodo better than a chloro, see Fig. (4b)) occupies the third hydrophobic pocket, as shown by computational docking simulation [162], and unpublished X-ray crystallographic data of the authors. The antitumor activities of the series have not yet been published.



- a) R = 7-I, $K_d = 0.067 \mu\text{M}$; $\text{IC}_{50} = 0.42 \mu\text{M}$
 b) R = 7-Cl, $K_d = 0.083 \mu\text{M}$; $\text{IC}_{50} = 1.53 \mu\text{M}$

Fig. (4). Representative 1,4-benzodiazepine-2,5-dione MDM2 inhibitors.

Indirect and Direct Structure-Based Design and Discovery of p53-MDM2 Inhibitors

The discovery of various classes of ligands that competitively bind at the N-terminal p53 binding site on MDM2, and the X-ray crystallographic elucidation of the

three dimensional (3D) structure of the p53-MDM2 binding interface [53], have engendered attempts at computer-aided structure-based and other rational drug design approaches to the discovery of small molecule inhibitors as will be outlined below, as well as our own unpublished MDM2 structure-based drug design research.

The binding interaction between MDM2 and p53 at the p53 transactivation domain and the MDM2 N-terminal domain has been quite well characterized both biochemically and biophysically [34, 35, 53, 164, 165]. The N-terminal domain of MDM2 (residues 2–125) binds to the p53 transactivation domain (residues 15–29). The MDM2 N-terminal domain has a unique fold with an extensive hydrophobic cleft at its center, with an accessible surface area of about 1400 sq. angstroms, which tightly binds the amphipathic α -helical transactivation domain of p53 with a K_d of 500 nM. The interaction involves the binding of the p53 α -helix to a hydrophobic cleft with a basin-like structure created by three α -helices from MDM2; helices 2, 3 and 4 (PDB ID 1YCR [53]) as depicted in Fig. (5).

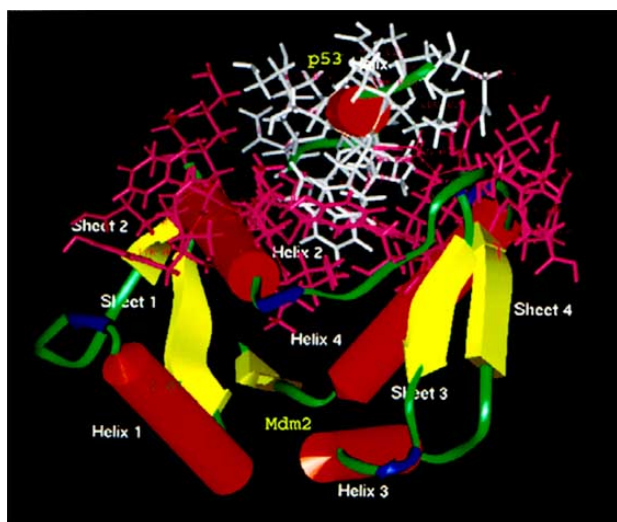


Fig. (5). p53 transactivation domain peptide in complex MDM2 N-terminal domain (PDB ID 1YCR). Amino acid residues of the p53 peptide are shown as white sticks while the MDM2 residues in contact with p53 are shown as dark gray.

There are three prominent hydrophobic pockets within the p53-binding site on MDM2 that are occupied by the hydrophobic side chains of p53 residues Phe19, Trp23 and Leu26. It appears that occupation of all three pockets is required for highly potent inhibitors. The design of small

molecules has focused on these three hydrophobic pockets for the minimum occupancy requirements that small molecule inhibitors must meet to possess high affinity binding to the site. Several hydrophobic groups, especially phenyl, *para*-chlorophenyl, *para*-bromophenyl and *para*-iodophenyl groups, have been shown to be suitable occupants. The specific moieties may vary depending on the scaffold employed to anchor the groups.

A quantitative structure-activity (QSAR) study was conducted on a limited set of high-affinity MDM2 inhibitory peptides using electrostatic and hydrophobic property-based descriptors generated with the HINT program [166]. The pharmacophoric features derived from this set of peptides was subsequently used to formulate a search query that was used to search the NCI chemical database to discover a small molecule pyrazolidinedione sulfonamide derivative lead compound (see Fig. (6a)), which represent a novel class of p53-MDM2 binding inhibitors [167]. The pharmacophore and database search query used is shown in Fig. (6b). The distance restraints were defined with a 20 % tolerance.

The binding mode of this compound on MDM2 is yet to be determined. However, from the binding modes observed for other small molecule inhibitors [146, 162], the two phenyl substituents on the pyrazolidinedione nitrogens might be occupying the two hydrophobic pockets occupied by Trp23 and Leu26, while possibly the *para*-isopropoxybenzoyl substituent binds in the third hydrophobic pocket occupied by p53's Phe19. This compound exhibited moderate affinity, with an IC_{50} of about 15 μ M in competition against peptide binding to MDM2, and was shown to cause an increase in p53 transcriptional activity. The results from optimization of this novel lead compound are awaited.

A *de novo* structure-based design approach based on the human p53-MDM2 complex structure reported by Kussie *et al.* [53] was used by Zhao *et al.* [168] to synthesize and test a series of new norbornane (8,9,10-trionorbornane) derivatives designated a syc compounds (also referred to as polycyclic compounds in the literature) [31, 158]. These compounds had hydrophobic groups attached to the norbornane scaffold, and intended to take advantage of the hydrophobic pockets occupied by p53 residues Phe19, Trp23 and Leu26. Among 23 synthetic targets tested by an ELISA method, five compounds, syc-7, syc-8, syc-11, syc-12 and syc-13 possessed concentration-dependent affinity for MDM2. These compounds also showed moderate growth inhibitory activity against MCF-7 (breast), NCI-H446 (small cell lung), HCT-8 (colon) and HeLa (cervical) cancer cell lines, in an MTT

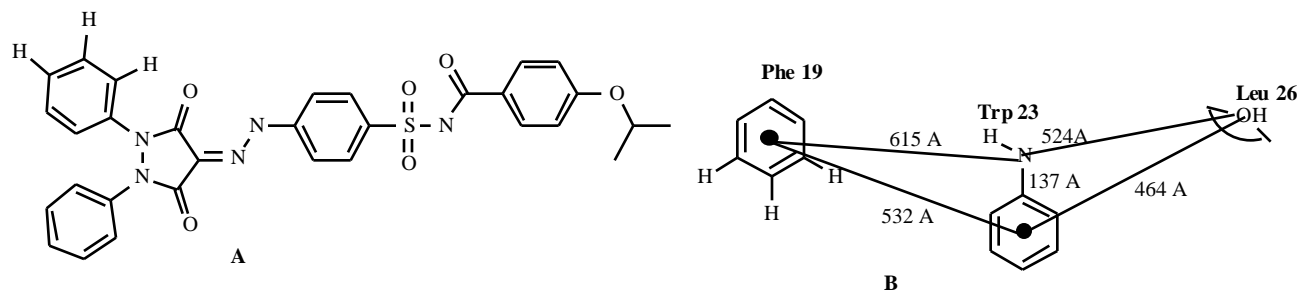


Fig. (6). A) The lead compound identified from database searching exercise. B) Pharmacophore query derived from peptide QSAR and used to search the NCI chemical database for potential MDM2 inhibitors.

assay, at 5 $\mu\text{g}/\text{ml}$, with % inhibition ranging from 50% to 80%. The compound designated syc-7 (2-benzoyloxy-3-hydroxy-5,6-(N,N'-diphenyl)-carboxamide-dicyclo[2.2.1]-heptane, Fig. (7)) showed about five fold selectivity between MCF7 cells and NEC normal cells, and also stimulated p53 and p21 accumulation, and induced apoptosis.

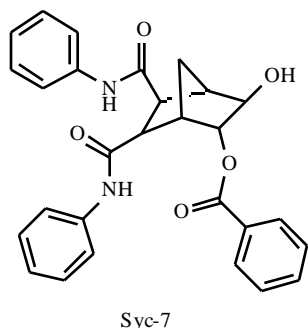
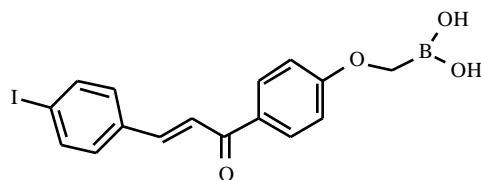


Fig. (7). Representative of structure-based designed norbornane derivative MDM2 inhibitory compounds.

Kumar *et al.* [169] have reported the synthesis and testing of novel chalcones boronic acids as inhibitors of p53-MDM2 binding. These new chalcones were designed based



Cell line:	MDA-MB-435	MDA-MB-435	MCF-7	MCF-10A	MCF-12A
IC ₅₀ (μM):	18	11	9.5	38	100

Fig. (8). Chalcone boronic acid designed on the bases on NMR data on binding interactions of chalcone carboxylic acids [Fig. (1), 160]. The IC₅₀ values are shown for the inhibition of the proliferation of various human breast cancer cell lines and a normal breast cell line (MFC-12A^a is a normal cell line).

on the NMR data reported by Stoll *et al.* [160], which indicated that the carboxylic acid group of the chalcones could be placed near the base of Lys51, to form a salt bridge. From the presumption that the acid group of the chalcone forms a salt bridge with this Lys51, and simultaneously breaking the salt bridge it forms with Glut25, it was envisioned that a boronic acid analogue might form a stronger salt bridge with Lys51 than the carboxylic acid analogue. In support of this notion, the boronic acid analogues exhibited a higher binding affinity relative to the original chalcones, and inhibited breast cancer cell growth with IC₅₀s in the 3.5-23 μM range. They were less cytotoxic to normal breast cell lines (IC₅₀s of 11-100 μM), indicating a modest selectivity against cancer cells relative to normal cells (see Fig. (8)).

High-affinity binding tryptophan derivatives that can be classified as small molecules were also recently designed to take advantage of the binding locus of p53's Trp23 on MDM2 [168] as shown in Fig. (9)). Anticancer activity of the series is not published.

Recently, with an assay to identify compounds that suppress tumor growth in a p53-dependent manner, a small molecule inhibitor has been identified called RITA (2,5-bis(5-hydroxymethyl-2thienyl)furan, (Fig. (10)), that is

purported to bind rather to p53 and disrupt p53-MDM2 binding [170]. It is thought that binding of RITA to p53 changes its conformation at the N-terminal that renders it incapable of binding to MDM2. This compound exhibited strong p53-dependent tumor growth suppression.

Targeting other p53-MDM2 Interactions with Small Molecules

In addition to the binding of p53 to MDM2 at the N-terminal domain, other domains of the MDM2 oncoprotein interact with p53. For example, as already discussed, the RING finger domain of MDM2 interacts with p53 as an E3 ligase to ubiquitinate p53 and target it for proteasomal destruction [61]. This interaction has also been shown to be amenable to inhibition by small molecules. Thus, Lai *et al.* [171], have demonstrated that the three small molecule compounds, an anilidosulfonamide, a bis-(amidinophenyl)-urea and a benzoylimidazolone (see Fig. (11)) can inhibit the E3 ligase activity of MDM2, and antagonize p53 ubiquitination. This area is very much underdeveloped, and provides an opportunity for identifying agents that may synergize with the p53-MDM2 binding inhibitors in multi-pronged approaches to targeting MDM2 for cancer therapy.

CONCLUSION AND PERSPECTIVES

The disruption of the p53-MDM2 interaction with small molecule inhibitors has been validated a potentially viable and attractive cancer therapeutic strategy. Current drug discovery efforts in this area are focused on the binding of p53 to the N-terminal domain of MDM2, which has been extensively characterized at the molecular level. There is very

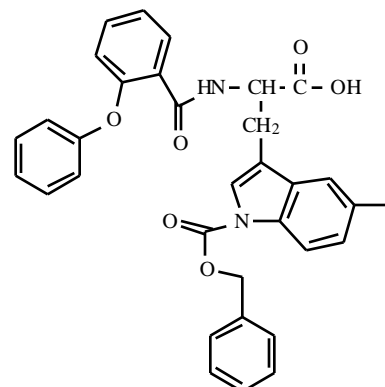


Fig. (9). Tryptophan derivative p53-MDM2 binding inhibitor [163].

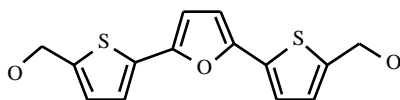


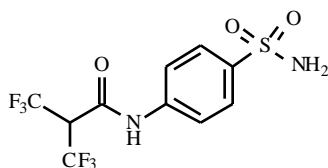
Fig. (10). RITA (2,5-bis(5-hydroxymethyl-2-thienyl)furan, a new inhibitor of p53-MDM2 binding that binds to the N-terminal domain of p53.

little information on small molecules targeting other regions of MDM2, like the RING finger domain. Moreover, there are still many untapped opportunities to discover small molecule inhibitors of MDM2. As detailed in this review and by Zhang and Zhang in this issue [44], there is a plethora of other proteins besides p53 that interact with MDM2. Many of those interactions enhance MDM2's ability to downregulate p53, and their interaction with MDM2 should be explored as novel anticancer targets. These targeting interactions may provide alternative or complementary approaches to targeting the p53-MDM2 interaction.

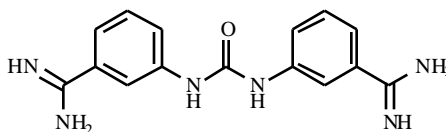
The sumoylation of MDM2, which is carried out by the Ubc9 protein to stabilize it and enhance its p53 degradation activity [76, 77], is one such example. Other examples can

multiple cancer therapeutic modalities (see reviews by Rayburn *et al.* [43], Bianco *et al.* [144] and Zhang *et al.* [42] in this issue). At the very least, MDM2 antagonists will be effective chemosensitization and/or radiation sensitization agents in treatment of tumors expressing functional p53, which constitute approximately half of all human cancers [175].

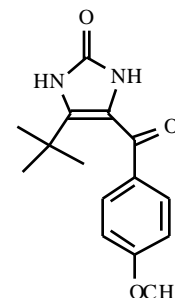
It should be noted that although MDM2 overexpression is oncogenic and indicates poor prognosis most situations, there are certain cases where overexpression of MDM2 may be associated with good prognosis. For example, MDM2 overexpression has been shown correlate with favorable prognostic parameters in estrogen receptor alpha positive breast cancer [176]. It has also been shown that in certain situations MDM2 may serve to suppress tumor growth rather than promote it, such as a case in malignant melanoma, where it was shown that the insulin-like growth factor 1 receptor (IGF-1R), an oncogenic receptor tyrosine kinase can be downregulated by MDM2-mediated ubiquitination targeting it for proteasomal degradation [177]. Thus, there may be some caveats to using MDM2 inhibitors in cancer therapy, but in the big picture, they may prove to be very useful novel anticancer agents.



N-sulfonamidoanilido-2,2-bis-(trifluoromethyl)acetamide



N,N'-bis-(diamido phenyl)urea



4-(p-methoxybenzoyl)-5-t-butylimidazo-2-one

Fig. (11). Small molecule inhibitors of the E3 ligase activity of MDM2 towards p53.

be found in the p53-independent oncogenic activities of MDM2, which include (i) its binding to, and destabilization of the cell cycle arresting, cyclin-dependent kinase inhibitor, p21WAF1/CIP1 [123], (ii) its binding to the p53 homologue p73, (iii) its destabilization of the HAT protein TIP60 involved in the induction of apoptosis, (iv) its destabilization of Numb, and (v) its positive modulation of the HIF-1 transcription factor. Further, MDM2 also inactivates the tumor suppressor Rb gene product [172], and stimulates the E2F1/DP1 transcription factors [173], resulting in the promotion of the G1 to S phase transition in cell cycle progression. It has also even been shown to confer a growth advantage to cells, in the absence of p53 and pRb, causing them to overcome a G1-cell cycle block induced by p107 [174]. These examples are given to illustrate the numerous opportunities still remaining that could be investigated for anticancer drug discovery targeting MDM2.

The drug development process for small molecule MDM2 inhibitors is still at an early preclinical stage, and as such, none has advanced into even Phase I human clinical trials. Proof-of-principle studies have been conducted in rodents [146] but primate studies have not yet been reported. This fledgling stage notwithstanding, targeting MDM2 holds a lot of promise for future clinical applications in

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