MDM2 Inhibitors for Pancreatic Cancer Therapy

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Abstract: MDM2 protein negatively regulates p53 and is found to be elevated in cancer cells. An attractive approach towards targeting MDM2 is the use of small molecule inhibitors that bind to MDM2 and disrupt the MDM2-p53 interaction. Our laboratory has been at the forefront in testing MDM2 inhibitors in pancreatic adenocarcinoma (PaCa), a deadly disease with ~50% wild-type p53 population. Emerging evidence suggests that apart from regulating p53, MDM2 can influence other key molecules involved in cancer. This review summarizes recent advancements in the development of MDM2 inhibitors, their novel primary and secondary targets and highlights their potential as therapeutics for PaCa.

Keywords: MDM2 and p53, small molecule inhibitors, pancreatic cancer.

P53 AND ITS SIGNIFICANCE

The tumor suppressor protein p53 is a transcription factor that has an essential role in regulating the cells response to various stresses through induction of cell cycle arrest, apoptosis or senescence [1, 2]. In response to numerous stresses such as DNA damage, UV irradiation and oncogenic signaling activated p53 binds DNA and regulates expression of several genes including the proapoptotic Bax, Puma and Noxa (Fig. **1**). P53 regulates WAF1/CIP1 encoding for p21. p21(WAF1) binds to the G1-S/CDK (CDK2) and S/CDK complexes (molecules important for the G1/S transition in the cell cycle) thus inhibiting their activity [3-5]. When p21(WAF1) is complexed with cdk2 the cell cannot pass through to the next stage of cell division. In addition, p53 has many anticancer mechanisms, and plays a role in apoptosis, genetic stability, and inhibition of angiogenesis (Fig. **1**). Loss of p53 function plays a significant role in tumor evolution by allowing evasion from p53-dependent responses [6]. p53 inactivation in tumors maybe through multiple mechanisms including, the inactivation of p53 function by point mutations in p53 itself or through the partial abrogation of signaling pathways or effector molecules that regulate p53 activity [7, 8]. However, numerous genetic studies have established that the restoration of p53 activity in established tumor cells is an exceptionally effective intervention [9].

MDM2 AND ITS SIGNIFICANCE

The p53 function is under tight regulation by its primary cellular inhibitor, the murine double minute 2 (MDM2; HDM2 in humans) (Fig. **1**). MDM2 was initially discovered as the product of an oncogene found overexpressed by amplification in a spontaneously transformed mouse cell line [10, 11]. MDM2 is an essential regulator of p53 in normal cells, but its deregulated expression provides growth advantage to cancer cells. Over expression of MDM2 due to the amplification of the *MDM2* gene was first found in sarcomas retaining wild-type p53 [12, 13], and this amplification was later observed in several other human cancers [14, 15]. Soon after its discovery, MDM2 was shown as a negative regulator of p53-mediated transactivation [16]. MDM2 and p53 regulate each other through an auto regulatory feedback loop. MDM2 gene contains a p53 promoter and is therefore transcriptionally regulated by p53. In this manner p53 itself regulates MDM2 at the level of transcription. Upon activation, p53 transcribes the *MDM2* gene and, in turn, the MDM2 protein inhibits p53 activity [17]. MDM2 also exports p53 out of the nucleus, promoting its degradation and rendering it inaccessible to the target genes [18, 19] and promotes proteasome-mediated degradation of p53 by functioning as an E3 ubiquitin ligase [20]. In this manner, MDM2 functions as an effective inhibitor of p53 activity. Recent genetic studies have shown that the loss of p53 induces tumor formation in mice, whereas its restoration leads to a rapid regression of established in situ tumors, further showing the cancer-therapeutic potential of p53 restoration [21]. Besides MDM2, MDM4 (also known as HDM4, MDMX or HDMX) and ARF (also known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice) also have an important role in controlling p53 stability [22, 23]. MDM4 is a structural homologue of MDM2 that can form a complex with MDM2 and potentiate the ubiquitylation of p53, and ARF is a tumor suppressor that interacts with MDM2 and inhibits p53 degradation, thereby stabilizing it [24] (only MDM2 will be the focus of this review). In more than half the tumors with defective p53 pathway, *TP53* itself is not mutated but the p53 pathway is abrogated. Mechanisms that result in this abrogation include increased expression of the p53-negative regulators MDM2 and MDM4 and deletion or epigenetic inactivation of the p53-positive regulator and MDM2 inhibitor ARF. Over the last two decades, a number of distinct therapeutic strategies have been pursued to restore p53 function for cancer treatment [2, 25-27]. Because the interaction between

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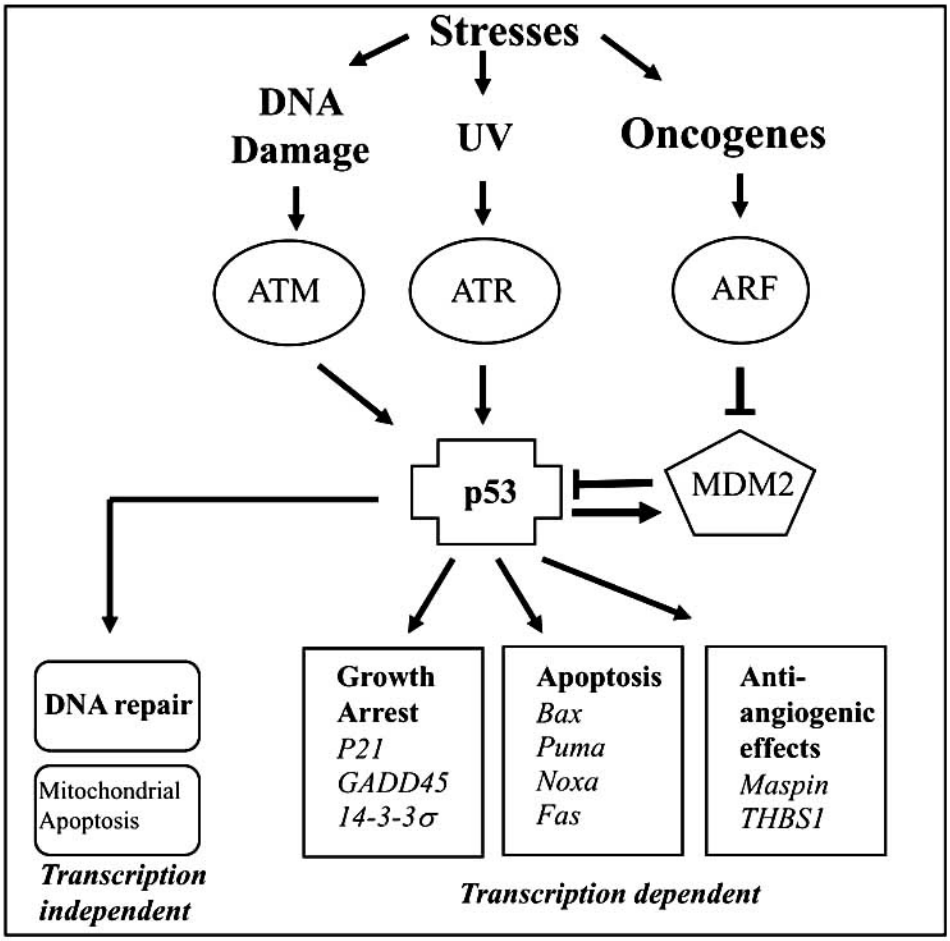

Fig. (1). The p53 pathway and its regulation by MDM2. In response to numerous stresses such as DNA damage, UV irradiation and oncogenic signaling p53 is transcriptionally activated. This results in activation of multiple pathways that are both transcription dependent and independent. MDM2 is a cellular regulator of p53 that bind to p53 and blocks effect p53 activation. MDM2 itself is under a negative feedback control of p53. (ATM ataxia telangiectasia mutated; ATR ataxia telangiectasia and Rad2 related; ARF alternate reading frame.

MDM2 and p53 is a primary mechanism for inhibition of the p53 function in cancers retaining wild-type p53, targeting the MDM2-p53 interaction by small molecules to reactivate p53 has emerged as a promising new cancer therapeutic strategy, and is the focus of this review.

REACTIVATING THE WILD-TYPE P53

Inhibition of p53 activity in tumors by the increased expression of MDM2 has been the target of development for many small-molecule-, peptide- and aptamer-based therapies [28]. MDM2 is over expressed in many human tumors, often owing to an amplification of a chromosome segment that includes *MDM2*, although over expression of the protein is possible without gene amplification [29-35]. There has been extensive validation of MDM2 as a target, ranging from studies with aptamers and peptides through to antisense approaches and, perhaps most significantly, was described in a path breaking study using a hypomorphic allele of *Mdm2* in the mouse [36]. In this study, nominal reductions in MDM2 levels were found to be sufficient to trigger a mild p53 response (as shown by increased levels of lymphopenia and apoptosis in intestinal crypts) in response to increased p53 activity. The volume of the thymus is also reduced and there is a small effect on weight gain during development. Gene dosage studies have found levels of MDM2 that selectively inhibit the development of colon carcinoma induced by the absence of adenomatous polyposis coli (*APACA*) without adverse affects on normal tissues. These powerful studies provided proof of a therapeutic index for MDM2 inhibition that has now been confirmed by the first small molecule candidates, including nutlin [37], MI-219 [38] and reactivation of p53 and induction of tumor cell apoptosis (RITA) also known as NSC 652287 [39], which produce tumor regression *in vivo* in human tumor xenografts in nude mice.

Non-Peptidic Small-Molecule Inhibitors of MDM2-p53 Interaction

The progress in the design of nonpeptidic, small-molecule inhibitors of the MDM2-p53 interaction (mentioned herein as MDM2 inhibitors; sometimes also called as HDM2 inhibitors) proceeded very slowly for almost a decade after the publication of the crystal structures. The very first class of bona fide, potent, nonpeptidic, small-molecule MDM2 inhibitors, known as Nutlins, was reported in 2004 [40-43]. The Nutlins contain a cis-imidazoline core

structure and one analogue, Nutlin-3, has potent *in vivo* antitumor activity in xenograft models of human cancer-retaining wild-type p53. The discovery of the Nutlins provided the important proof-of-concept and fueled enthusiasm for the design and development of small-molecule MDM2 inhibitors. In the last 4 years, several new classes of small-molecule MDM2 inhibitors have been discovered using different approaches [44-46]. Using a computational structure-based de novo design strategy, a new class of spiro-oxindoles that are potent inhibitors of MDM2 [47], as exemplified by MI-63 and MI-219 were designed. In this regard Nutlin-3 a cis-imidazole has been well studied in different cancers. Our MI series of MDM2 inhibitors belong to different class (spiro-oxindole) and have a slightly higher affinity towards MDM2 when compared to Nutlins. Using a structure-based de novo design strategy it was shown that the interaction between p53 and MDM2 is primarily mediated by four key hydrophobic residues (Phe 19, Leu 22, Trp 23 and Leu 26) of p53 and a small but deep hydrophobic cleft in MDM2. Nutlin-3 mimics the interactions of the p53 peptide to a high degree, with one bromophenyl moiety sitting deeply in the Trp pocket, the other bromophenyl group occupying the Leu pocket, and the ethyl ether side chain directed toward the Phe pocket. In essence, the imidazoline scaffold replaces the helical backbone of the peptide and is able to direct, in a fairly rigid fashion, the projection of three groups into the pockets normally occupied by Phe19, Trp23, and Leu26 of p53. However, unlike Nutlin-3 in case of our inhibitors (MI series), computational modeling predicted that MI-219 mimics the four (instead of three in case of Nutlin-3) key binding residues in p53 (Phe-19, Leu-22, Trp-23 and Leu-26) resulting in optimal hydrogen bonding and hydrophobic interactions with MDM2. Both nutlins and MI-219 enter many types of cultured cells and inhibit the p53–MDM2 interaction with a high degree of specificity, leading to the stabilization of p53 and the activation of the p53 pathway [48]. Proliferating cancer cells that express wild-type p53 are effectively arrested in the G1 and G2 phases of the cell cycle or can undergo apoptosis when treated with micro molar concentrations of nutlins [48]. This indicates that some cells are more susceptible to nutlin-induced apoptosis than other cells in which a reversible cell cycle arrest is observed. The key cellular characteristics that underlie this difference in response are the subject of intense investigation. This differential response may occur owing to abnormalities further downstream in the p53 pathway. Other small molecules that have been developed to target the p53–MDM2 interaction include benzodiazepenes [49]. The benzodiazepene-based derivatives disrupt the MDM2–p53 interaction *in vitro* with IC_{50} values of 0.5-2 μM and have also been shown to suppress the growth of cell lines containing wild-type p53. Administration of the benzodiazepene derivative TDP665759 to normal mice led to an increase in p21 (also known as WAF1 and CIP1) levels in liver samples [50]. Finally, TDP665759 synergizes with doxorubicin both in culture and in xenografts of A375 melanoma cells to decrease tumor growth.

With so many targets identified against the MDM2-p53 interaction and supporting preclinical laboratory evidence it is imperative that effective MDM2 inhibitors will become a major form of therapy in the coming years. However, several potential drawbacks to targeting the MDM2–P53 interaction can be envisioned. First, MDM2 is induced by p53 activation as part of an inducible feedback loop that negatively regulates the p53 response. Therefore, the drugs would induce their target, limiting their potential efficacy. Second, the current molecules fail to effectively target MDM4. The binding pocket of the N terminus of MDM2 has shown itself to be eminently druggable, and a future challenge is whether or not these drugs can proceed to the clinic and whether they can also be refined to target other MDM2 family members such as MDM4. Apart from this avenue of research, other target sites have been identified in this p53 regulatory pathway that show the potential for drug development, and it remains to be seen if they generate therapeutic leads that have low toxicity in normal tissues.

Compounds that Target p53 Regulators

Activated p53 is under multiple post-translational control that includes acetylation, methylation, phosphorylation, neddylation and sumoylation [1, 51-59]. Activating p53 using small may not be sufficient for proper p53 function and combinations with agents that suppress post-translational p53 blockers such as acetylation would benefit the overall outcome of such therapy. An example of this type of agent which was identified through a p53-based phenotypic screen are tenovin-1 and its more water-soluble derivative tenovin-6 [60, 61]. Tenovins rapidly increase p53 levels in cells treated with low micro molar concentrations, and daily intraperitoneal injection of tenovin-6 at 50 mg per kg delays xenograft tumor growth in mouse models [52]. Through a yeast genetic screen and subsequent enzymatic assays tenovins were shown to inhibit the NAD^+-dependent deacetylase activity of SIRT1 and SIRT2 [62, 63], two members of the sirtuin family of class III histone deacetylases. p53 deacetylation by SIRT1 impairs p53 stability and transcriptional activity. Therefore, inhibiting the sirtuins should lead to increased p53 stability. Indeed, treatment of MCF-7 cells with tenovins led to the accumulation of acetylated p53 and acetylated tubulin, which are established substrates of SIRT1 and SIRT2, respectively. Further chemical optimization of the potency of the tenovins is now possible owing to the elucidation of SIRT1 and SIRT2 as the cellular targets. The discovery and characterization of the tenovins is an example of how current technological advances in target identification and p53 basic research contribute to the understanding of the mechanism of action of bioactive small molecules.

Therapeutic Potential of MDM2 Inhibitors in Different Cancers

Over the last several years different MDM2 inhibitor have been tested in multiple tumor systems with some success. These inhibitors have been shown to induce cell growth arrest and apoptosis *in vitro* as well tumor growth arrest in different animal model systems. Studies referring to the use of MDM2 inhibitors in individual cancers are given below:

Prostate Cancer

Prostate cancer is a deadly disease with an estimated 186,320 new cases and 28,660 deaths in the year 2008 in the

United States alone [64] , and currently ranks the second most frequent cause of cancer related death in males in the United States. The slow and unsatisfactory progress for prostate cancer treatment warrants novel preventive and/or therapeutic approaches. Numerous laboratories have demonstrated that nutlin-3 as to have antitumor activity against prostate cancer cells expressing both wild-type and mutant p53 [65-67]. In an important study the antiproliferative function for nutlin-3 was screened on a panel of prostate cancer cells that have distinct p53 and AR status [66]. LNCaP cells contain wild p53, PC3 cells are p53 null due to a single base deletion in codon 138 that prevents expression, and DU145 cells express mutant p53 that harbors missense mutations in codons 223 and 274. In the same study it was found that the proliferation of LNCaP cells alone was markedly reduced by nutlin-3. LNCaP cells underwent a G2/M phase cell cycle arrest, with a corresponding reduction in the number of cells in S phase. Consistent with these observations, it was shown that p21 levels are increased in LNCaP cells in response to nutlin-3, but not in DU145 or PC3 cells. Further these studies also prove that nutlin-3 causes apoptosis only in LNCaP cells which appears to involve activation of caspase-3 and upregulation of the p53- responsive gene PUMA. Because all three of the cell lines express the nutlin-3 target MDM2 but only LNCaP cells express functional p53 one might assume that wild-type p53 is required for nutlin-3 to generate its effects on proliferation of prostate cancer cells. However, it was observed that nutlin-3 reduces steady-state AR protein levels in LNCaP cells. This is important because androgen signaling is not only required for the proliferation of LNCaP cells but also the development and maintenance of human prostate tumors. Previous studies have demonstrated that AR depletion in LNCaP cells results in inhibition of proliferation. Therefore it was proposed that nutlin-3 might inhibit growth of LNCaP cells through not only p53 signaling but also by downregulation of androgen signaling. Increased levels of the MDM2 protein, which is known to target AR for destruction, could account for the effects of nutlin-3 on AR levels.

Colon Cancer

The spiro-oxindole MI-43, was examined for its cellular mechanism of action and therapeutic potential in colon cancer [68]. In this report Shangary *et al*., have shown that MI-43 binds to MDM2 protein with a K(i) value of 18 nmol/L that is 300 times more potent than a native p53 peptide. Further, MI-43 blocks the intracellular MDM2-p53 interaction and induces p53 accumulation in both normal and cancer cells, with wild-type p53 without causing p53 phosphorylation. Induction of p53 leads to modulation of the expression of p53 target genes, including up-regulation of p21 and MDM2 in normal primary human cells and in colon cancer cells with wild-type p53. Using HCT-116 isogenic colon cancer cell lines differing only in p53 status or RNA interference to knockdown expression of p53 in the RKO colon cancer cell line, it was shown that the cell growth inhibition and cell death induction by MI-43 was p53 dependent. Furthermore, induction of cell cycle arrest by MI-43 is dependent on p53 and p21. In normal cells, MI-43 induces cell cycle arrest but not apoptosis. In the same study it was shown that the inhibitor could induce growth inhibition or apoptosis in mut-p53 but at very high concentrations (> 20 μM). Although the mechanism was not explored yet it can be speculated that at such higher concentrations these inhibitors may reactivate the p53 sister pathways such as p73 and p63 that are also known inducers of apoptosis utilizing similar mechanisms and are rarely mutated in cancers. This study suggests that p53 activation by a potent and specific spiro-oxindole MDM2 antagonist may represent a promising therapeutic strategy for the treatment of colon cancer and should be further evaluated *in vivo* and in the clinic.

MDM2 Inhibitors in Blood Malignancies

Blood malignancies, such as acute myeloid leukemia (AML), B-chronic lymphocytic leukemia (B-CLL), and multiple myeloma (MM) are potentially attractive tumor types for MDM2 inhibitor-based therapy due to the rarity of p53 mutations in these tumor models. Ex vivo experiments using AML, B-CLL, and multiple myeloma patient specimens have indeed shown that inhibition of MDM2 by Nutlin-3 and MI-63 effectively triggers apoptosis. Conclusive evidence of the dependence of the activity of MDM2 inhibitors upon p53 status in B-CLL was provided by a recent study using MI-63 and Nutlin-3 in a cohort of more than 100 B-CLL patients [69]. Nutlin-3 synergizes with doxorubicin and cytosine arabinoside in killing myeloblasts in AML and with doxorubicin, chlorambucil, and fludarabine in killing leukemic cells in B-CLL patient specimens [70-72]. Importantly, both the single agent and the combination effect of Nutlin-3 are selective for cancer versus normal cells, as revealed by the lack of toxicity to peripheral blood mononuclear cells or bone marrow-derived hematopoietic progenitors and bone marrow stromal epithelium cells [73, 74]. Our groups has extensively studied MI-319, MI-219 and Nutlin-3 in Hodgkin's and non Hodgkin's lymphoma [75]. Our cell growth inhibition and gene expression profiling experiments revealed that the three compounds have quite similar potency against the tumor cell lines tested in this study. *In vitro*, MI-319 exhibited the strongest anti-proliferation activity against FSCCL and four patient cells, which all have wild-type p53. Western blotting, cell cycle and apoptosis analysis experiments indicated that FSCCL exhibited strong cell cycle arrest and significant apoptotic cell death; cells with mutant p53 did not show significant apoptotic cell death with drug concentrations up to 10 μM, but displayed weaker and differential cell cycle responses. In our systemic mouse model for FSCCL, MI-319 was tolerated well by the animals, displayed effectiveness against FSCCL-lymphoma cells in blood, brain and bone marrow, and achieved significant therapeutic impact ($p < 0.0001$) by conferring the treatment group a > 28% (%ILS, 14.4 days) increase in median survival days.

MDM2 Inhibitors in Rhabdosarcoma

Rhabdomyosarcoma (RMS) is a type of cancer of connective tissues, in which the cancer cells are thought to arise from skeletal muscle progenitors. It can also be found attached to muscle tissue, wrapped around intestines, or anywhere, to exclude the neck area. Interestingly seventy to eighty percent of RMS tumors retain wild-type p53 making them a logical target for MDM2 inhibitor based therapies. To this end it was shown that Nutlin-3 could effectively restore p53 function in both normal MDM2 expression and MDM2

overexpression RMS cell lines with wild-type p53. Further a combination of vincristine or actinomycin D with nutlin-3 enhanced the antitumor activity in RMS cell lines with wild-type p53 [76]. In another recent study using our class of MDM2 inhibitor (MI-63) it was shown that induces apoptosis in embryonal and alveolar rhabdomyosarcoma cells with wild-type p53 [77]. In the same study it was shown that MI-63 or Nutlin-3 when combined with standard chemotherapeutics drug doxorubicin could enhance the efficacy of the individual inhibitors alone suggesting a possible clinical application of such potent combination for pediatric malignancies.

MDM2 Inhibitors in Pancreatic Adenocarcinoma PaCa

Pancreatic adenocarcinoma (PaCa) is a deadly disease with an annual mortality of about 35000 in United States [78]. PaCa is an inherently resistant cancer and is poorly amenable to current and standard chemotherapeutics such gemcitabine [79] and newer agent to target this intractable malignancy are urgently needed. So far no, studies have reported the effect of such inhibitors against PaCa and this may be due to lack of suitable cell lines (Capan-2 is the only wt-p53 PaCa cell line). To this end our laboratory is the first to show the growth inhibitory and apoptotic potential of MDM2 inhibitors MI-319, MI-219 and Nutlin-3 in PaCa (results on the pathways affected in PaCa are summarized in Table **1**). These inhibitors specifically induced cell growth inhibition and apoptosis in wt-p53 PaCa cells. Growth inhibition and apoptosis by MDM2 inhibitors was accompanied by increase in levels of p53 along with $p21^{WAF1}$ and the proapoptotic Puma. SiRNA against $p21^{WAF1}$ abrogated the growth inhibition of PaCa cells confirming $p21^{WAF1}$ as a key player downstream of activated p53. Immunoprecipitation-western blot analysis revealed reduced association of MDM2-p53 interaction in drug exposed PaCa cells (Table **1**). Further, using wt-p53 xenograft of Capan-2, we found that oral administration of MI-319 at 300 mg/kg for 14 days resulted in significant tumor growth inhibition without any observed toxicity to the animals. No tumor inhibition was found in mut-p53 BxPC-3 xenografts. We also explored combination treatments of these inhibitors with chemotherapeutics such as gemcitabine and cisplatin. Gemcitabine is the only approved chemotherapeutic for PaCa, yet has modest impact on the inherently resistant disease PaCa [80]. Interestingly MI-319, MI-219-gemcitabine combination worked specifically enhanced cell growth inhibition and apoptosis in PaCa cells with wt-p53 suggesting that this potent combination can be used in the clinic for wt-p53 population in this dreadful disease. We also tested the combination of MI-319 with cisplatin which although less popular for PaCa yet is a potent p53 inducer. Apart from being a potent combination in wt-p53 PaCa tumors most interestingly MDM2 inhibitor-cisplatin combination drastically induced growth inhibition apoptosis and tumor growth inhibition in mut-p53 PaCa cell lines and xenografts respectively [81]. Mechanistically using siRNA silencing it was proven that the p53 family member p73 (which is rarely mutated in PaCa and other cancers) was found to be responsible for the observed anticancer/anti-tumor effects (Table **1**). That the combination worked in a p53 null system ($HCT116^{-/-}$) further reiterated that MI-319-cisplatin combination could effectively induce apoptosis through a p73 dependent mechanism. However, further in-depth mechanistic studies are warranted specifically in PaCa.

Molecular Mechanism of p53 Independent Functions of MDM2 Inhibitors

The availability of potent and specific MDM2 inhibitors, such as Nutlin-3 and MI-219, has provided the opportunity to examine in detail the molecular mechanism of p53 activation. In competition with a p53-based peptide, Nutlin-3 and MI-219 bind with high affinity to MDM2 (K_i = 36 and 5 nmol/L, respectively). They block the intracellular MDM2-p53 interaction and induce the accumulation of p53 and the activation of the p53 pathway in tumor and normal cells. Conventional genotoxic anticancer agents and radiation also induce the accumulation and activation of p53, but they do so by posttranslational modifications of p53, such as phosphorylation. In contrast, Nutlin-3 induces neither DNA damage nor p53 phosphorylation in cells. Hence, small-molecule MDM2 inhibitors represent a new class of nongenotoxic agents that can reactivate the p53 function. Apart from regulating p53 recent studies have shown p53 independent functions of MDM2. In this regard Zhang and co-workers have extensively reviewed a whole range on targets governed by MDM2 [82]. Targeting MDM2 by agents such as MI-219 or nutlin may affect a myriad of other key cellular molecules that play significant role in cell

Table 1. Preclinical Testing of MI Series of MDM2 Inhibitors in PaCa *In Vitro* and *In Vivo* (Subcutaneous Animal Tumor Model. GI (Growth Inhibition); SIRT1 (Sirtuin 1); MI-10 (Inactive Analog)

Agent	GI	Apoptosis	p53	p21	Tumor GI	p73	SIRT1	Ku70
MI-219	↑	↑	↑	↑	↑	↑	↓	↓
MI-319	↑	↑	↑	↑	↑	↑	↓	↓
MI-10	-	-	-	-	-	-	-	-
+Gemcitabine (wt-p53)	↑	↑	↑	↑	↑	↑	-	-
+ Oxaliplatin + Cisplatin (wt-p53 and mut–p53)	↑	↑	↑	↑	↑	↑	-	-

growth and apoptosis. In-depth mechanistic studies on the mode of action of inhibitors on MDM2 and the consequent p53 reactivation are lacking and it was of interest to us to explore the roles of crucial proteins that are involved in the regulation of p53. Activated p53 is known to be influenced by multiple post-translational control processes such as phosphorylation and acetylation that positively regulate p53 function [83]. Acetylation is an important epigenetic phenomenon in the biology of p53 [84, 85]. Upon stress, p53 is acetylated at Lys382 which enhances its DNA binding activity [86]. Moreover, deacetylation of p53 by SIRT1 has been shown to repress p53 mediated cell cycle regulation and apoptosis. SIRT1 is also known to deacetylate another protein Ku70 which, in turn, interacts with Bax and is responsible for blocking Bax entry into mitochondria. Therefore, we sought to determine whether acetylation of p53 could be influenced by our inhibitors in PaCa cells. Indeed our results showed that MI-219 treatment suppresses SIRT1 protein and simultaneously enhances acetylation of p53 (Table **1**). Using state of the art Surface plasmon resonance techniques we studied the binding between MI-319 or Nutlin-3 and Ku70 and our results confirm high affinity association between the two (Table **1**). Interestingly MI-219 treatment resulted in the suppression of Ku70 expression along with disruption of Ku70-Bax interaction (Table **1**). This observation is of great importance because it proves that MDM2 inhibitor not only blocks MDM2 which is its primary target but also suppresses two secondary targets the negative regulator 'SIRT1', which is a molecule that regulates p53 function and Ku70. Although it is too preliminary to confirm the true binding/interaction site of Ku70 or SIRT1 to MI-319, yet is can be speculated that MI-319 or Nutlin-3 may interact with peptide sequence (LSQETFSDLWKLL) similar to p53 transactivation domain towards which both Nutlin-3 or MI series of inhibitors were built.

As MI-219 does not alter MDM2 expression yet Ku70 and SIRT1 are suppressed suggesting that these drugs may have a MDM2 independent role in the biology of cells. However, compelling evidence in literature supports to a MDM2 dependent mechanism of action of these drugs on Ku70 and SIRT1. Our cell free FRET based SIRT1 activity assay showed inhibition of SIRT1 activity by MDM2 inhibitors. Yet in a cellular system the dynamics of SIRT1 is complex. Studies so far suggest that only wt-p53 can inhibit SIRT1 while cells that have lost or have mutations in p53 have over expressed SIRT1 and cannot repress it. This certainly points out that the suppression of SIRT1 in our system is p53 dependent however elucidation of the exact mechanism of action requires further work. As far as Ku70 is concerned, very recently Nutlin, a drug with similar mode of action as MI-219 was shown to disrupt hdm2-Ku70 interaction. Based on our results and those of others we propose multiple mode of action MI-219 on SIRT1 and Ku70. MDM2 inhibitors down regulates SIRT1 that in principle may prevent Ku70 and p53 deacetylation. Surface plasmon resonance and Co-IP results confirm that MI drugs directly bind to Ku70 as well as disrupt Ku70-Bax interaction (Table **1**). Although yet to be proved, it is suggested that such a di-

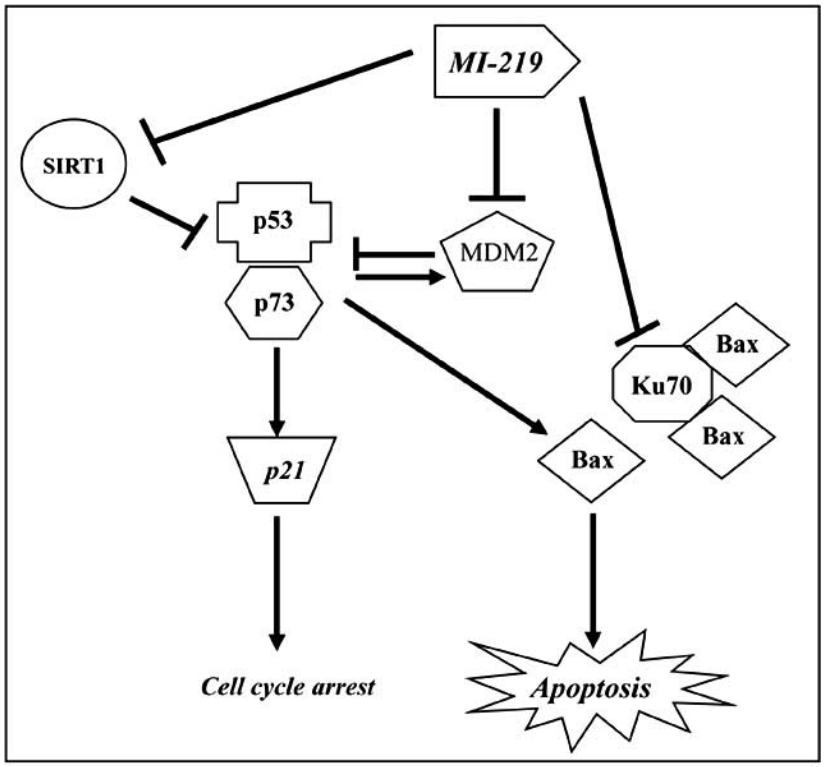

Fig. (2). Multiple secondary targets of MDM2 inhibitor MI-219. Activated p53/p73 and its affecter pathways such as Bax are under multiple post translational controls. SIRT1 is a histone deacetylase that keeps activated p53 under regulation through deacetylation. Ku70 is a component of homologous end joining DNA damage repair machinery that tightly binds to Bax and ultimately prevents apoptosis. MI-219 can not only target MDM2, it also downregulates SIRT1 and KU70 and disrupts the SIRT1-Bax and Ku70-Bax interactions.

Table 2. Studies of MDM2 Inhibitors in Different Cancers

Cancer Type	MDM2 Inhibitor	Reference
Prostate	Nutlin-3	[65-67]
Colon	MI-43, Nutlin-3	[68]
Blood Malignancies	MI-319, MI-219, MI-63, Nutlin-3	[69-75]
Rhabdosarcoma	Nutlin-3, MI-63	[76-77]
Pancreatic	MI-219, MI-319, Nutlin-3	[81]

rect binding may induce conformational changes in Ku70 rendering it ineffective in binding to Bax and therefore allowing the latter to induce apoptosis. MI drugs also directly suppress Ku70 mRNA and protein expression (Table **1**) which in turn allows p53 induced free Bax to mediate apoptotic events. A schematic diagram of the proposed and above discussed mechanism is given in Fig. (**2**).

Current Status of MDM2 Inhibitors in the Clinic

Although proven to be successful in the laboratory in multiple cancer models (summarized in Table **2**) MDM2 inhibitors or approaches that utilize reactivation of p53 have a long way to go before they are acceptable in the clinic. Molecules that reactivate the mut-p53 through protein conformational changes are currently in Phase I clinical trials a few examples are PRIMA (Phase I APR-246), CP-31398 (Phase I) and PhiKan-08 (Phase I). Small molecules that activate p53 through disruption of MDM2-p53 binding such as MI-219, Nutlin-3 are in phase I. RITA a p53 binding targeted agent and tenovin (SIRT1 inhibitor) are still in a pre-clinical testing phase. Leptomycin B (a CRM1 (Exportin 1) binding agent that mediates p53 reactivation) is in Phase I while Actinomycin D (an RPL11 and RPL5 (Ribosomal protein L) releasing agent) has been approved for Phase I [87]. Certain combinations such as nutlin with mitotic inhibitors for example BI-2536 (PLK1 (Polo-Like Kinase) inhibitor) or with VX680 (Aurora kinase inhibitor) are also in Phase I.

Impact and Clinical Relevance of MDM2 Inhibitors

It is without doubt that small molecule inhibitors that target MDM2 to reactivate p53 pathway function could have wide applications in the treatment of cancer. With the advent nutlin-3 and MI-219 that are potent and specific small-molecule inhibitors of MDM2 has certainly made a significant positive impact in this direction. Studies using these inhibitors in preclinical models have already provided strong evidence that targeting the MDM2-p53 interaction using small-molecule inhibitors is a promising cancer therapeutic approach. Clinical testing of these new agents should provide the ultimate proof for this therapeutic strategy. Both these inhibitors are now in late stage preclinical development and are expected to progress into human clinical trials over the next two years. Although initially designed to target only the wt-p53, yet studies from our laboratory in PaCa and emerging evidence from those of others support the fact that these inhibitors are effective on both wt-p53 and mut-p53 tumors. That these inhibitors can synergize with standard chemotherapeutics underscores their significance in the overall selectivity and therapeutic index against numerous tumors. In the sense that many other pathways in biology offer a similar challenge, the intense effort to target the p53 pathway is encouraging and supportive of the development of new approaches to drug discovery and therapy.

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