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Targeting p53 for Novel Anticancer Therapy[1]

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Abstract

Carcinogenesis is a multistage process, involving oncogene activation and tumor suppressor gene inactivation as well as complex interactions between tumor and host tissues, leading ultimately to an aggressive metastatic phenotype. Among many genetic lesions, mutational inactivation of p53 tumor suppressor, the "guardian of the genome," is the most frequent event found in 50% of human cancers. p53 plays a critical role in tumor suppression mainly by inducing growth arrest, apoptosis, and senescence, as well as by blocking angiogenesis. In addition, p53 generally confers the cancer cell sensitivity to chemoradiation. Thus, p53 becomes the most appealing target for mechanism-driven anticancer drug discovery. This review will focus on the approaches currently undertaken to target p53 and its regulators with an overall goal either to activate p53 in cancer cells for killing or to inactivate p53 temporarily in normal cells for chemoradiation protection. The compounds that activate wild type (wt) p53 would have an application for the treatment of wt p53-containing human cancer. Likewise, the compounds that change p53 conformation from mutant to wt p53 (p53 reactivation) or that kill the cancer cells with mutant p53 using a synthetic lethal mechanism can be used to selectively treat human cancer harboring a mutant p53. The inhibitors of wt p53 can be used on a temporary basis to reduce the normal cell toxicity derived from p53 activation. Thus, successful development of these three classes of p53 modulators, to be used alone or in combination with chemoradiation, will revolutionize current anticancer therapies and benefit cancer patients.

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Introduction

Cancer is usually associated with aberrant cell cycle progression and defective apoptosis induction due to the activation of proto-oncogenes and/or inactivation of tumor suppressor genes [1]. The evolving molecular events often provide the intervening candidate targets for the development of cancer therapy. One of the most promising targets is p53, a well-established and frequently mutated tumor suppressor in human cancer. Since its first discovery in 1979 as an oncogene [2,3], and particularly after its rediscovery as a tumor suppressor gene in 1989 [4,5], p53 has been the hot spot gene for cancer biologists seeking to elucidate the mechanisms of tumor formation and to validate it as a potential cancer therapy target [6–8].

It is well known now that p53 acts biochemically as a transcription factor and biologically as a powerful tumor suppressor. Under normal, unstressed conditions, p53 protein remains undetectable due to its short half-life. The p53 instability is primarily controlled by its negative regulator Mdm2, which, as an E3 ubiquitin ligase, targets p53 for proteasome-mediated degradation [9,10]. Other E3 ubiquitin ligases, which are also implicated in p53 degradation, are Pirh2 and COP1 [11,12]. Another source of p53 instability comes from its own physical property with a melting temperature slightly above body temperature [13]. p53 responds to a wide variety of cellular stresses including genotoxic damages, oncogene activation, and hypoxia [14,15] and is activated on posttranslational modifications by phosphorylation, acetylation, ubiquitination, and methylation [16–18]. Activated p53 then performs its two well-known biological functions: inducing apoptosis or inducing growth arrest [15,19]. The p53-induced apoptosis is mediated by the

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mitochondrial pathway through transcription-dependent or transcription-independent mechanisms and by the death receptor pathway through transcriptional activation of FAS and KILLER/DR5 [8,19,20]. p53 also transcriptionally represses cell survival genes such as *Bcl-2*, *survivin*, *IGFR*, *Mcl-1*, and *PIK3CA* [21–24] through multiple mechanisms [25]. Conversely, p53-induced growth arrest is mainly mediated through up-regulation of p21, Gadd45, 14-3-3σ, and PTGFβ among others, through a direct DNA binding and transactivation [8,26]. Other p53-involved anticancer mechanisms include induction of cellular senescence [27,28], inhibition of angiogenesis [29,30], and regulation of autophagy [31]. Although the major function of p53 is the "killer," p53 is also implicated in some cases as a "healer" to enhance the cell survival [21,32].

Given the central role of p53 in cancer prevention and suppression and in chemosensitization or radiosensitization, p53 has to be abrogated during carcinogenesis for most cancers to arise. Indeed, p53 is inactivated by point mutations in more than 50% of human cancers (see http://www.iarc.fr/p53) with a majority of mutations occurring in the DNA binding domain, which either change wt p53 conformation (conformation mutants, e.g., 175H, 249S, 281G) or abolish its DNA contact (contact mutants, e.g., 248W, 273H) [33]. Furthermore, in cancer carrying a wt p53, p53 is often nonfunctional as a result of either being degraded by overexpressed Mdm2 [9,10] or being excluded from the nucleus where p53 acts as a transcriptional factor [19,34,35]. In this review, we aimed to discuss various approaches 1) to activate wt p53, 2) to reactivate mutant p53 or selectively kill cancer cells with mutant p53, and 3) to temporarily inhibit wt p53 for normal cell protection (see Figure 1 and Table 1). Successful clinical development of these three classes of novel compounds would eventually revolutionize the current cancer therapies to benefit a majority of cancer patients.

Targeting p53 Itself

Targeting wt p53 — To Activate

The approaches include the use of chemoradiation to activate endogenous wt p53, of gene therapy to introduce wt p53 or modified adenovirus to kill cancer cells with mutant p53, and of synthetic peptides or nongenotoxic small molecules to activate endogenous wt p53.

Chemoradiation. Conventional anticancer therapies target p53 because almost all genotoxic anticancer drugs as well as ionizing radiation (IR) cause the substantial DNA damage, which triggers p53 activation and stabilization [36]. Early preclinical studies using both *in vitro* cell and *in vivo* tumor models showed that cells or tumors with a wt p53 are more sensitive to chemoradiation [37,38]. The early clinical studies further showed that mutant p53 confers chemoresistance in patients with ovarian cancer [39,40], breast cancer [41,42], gastric and colorectal

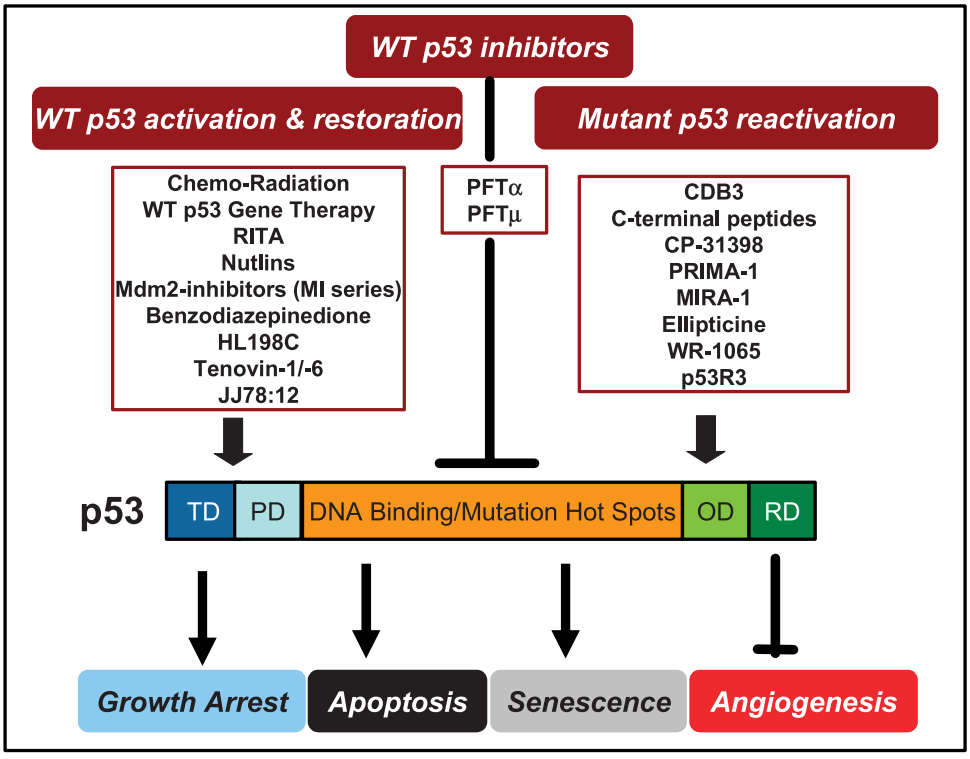
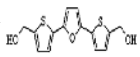
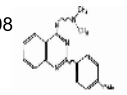
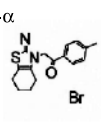
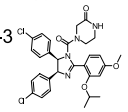
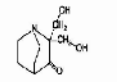
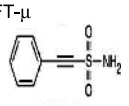
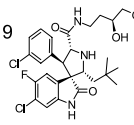
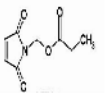
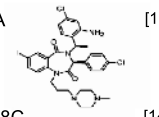
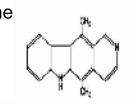
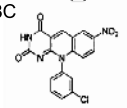
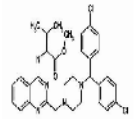
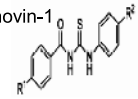
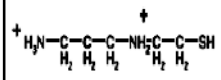
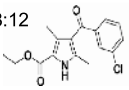


Figure 1. Current approaches for p53 targeting: p53, the "guardian of the genome," consists of 393 amino acids with four distinct functional domains. The transactivation domain (TD) and proline-rich domain (PD) is located at the N-terminus, the DNA binding and mutation hot spots domain at the central of the molecule, whereas the oligomerization domain (OD) and regulatory domain (RD) at the C-terminus. On activation, p53 plays a pivotal role in tumor suppression by inducing growth arrest, apoptosis, and senescence, as well as by blocking angiogenesis. Wild-type p53 also confers the sensitivity of cancer cells to chemoradiation. Thus, p53 becomes an appealing therapeutic target for anticancer drug discovery. As illustrated in the figure, three classes of p53 targeting compounds have been identified and characterized. The first class are the compounds that activate or restore wild-type p53 function and can be used in human cancers harboring a wt p53. The second class of compounds reactivates and rescues the mutant p53 with an application in human cancers carrying a p53 mutation. The third class is capable of inhibiting wt p53 and can be used during chemoradiation to block p53 activation in normal cells, thus reducing cytotoxicity.

Table 1. Structure of Small-Molecule p53 Activators, Reactivators, and Inhibitors.

WT p53 Activators			Mutant p53 Reactivators			WT p53 Inhibitors		
Name	Structure	Reference	Name	Structure	Reference	Name	Structure	Reference
RITA		[63]	CP-31398		[77]	PFT-α		[67]
Nutlin-3		[129]	PRIMA-1		[82]	PFT-μ		[68]
MI-219		[127]	MIRA-1		[83]			
BDA		[130]	Ellipiticine		[92]			
HLI98C		[146]	P53R3		[98]			
Tenovin-1		[158]	WR-1065		[97]			
JJ78:12		[159]						

cancer [43], and hematological malignancies [42]. Extensive follow-up studies in both preclinical and clinical settings showed that in general, cancer cells with a wt p53 are more sensitive to chemoradiation [44–47], but there are quite few exceptions. For example, breast cancer patients with a transcription-deficient mutant p53 had a better response to chemotherapy than patients with a wt p53 [48]. The same was true for MCF7 breast cancer cells and HCT116 colon cancer cells with p53 either abrogated or deleted, as a result of increased cellular vulnerability to G_2 checkpoint abrogators [49,50]. In multiple head and neck cancer cell lines, the absence of p53 appeared to be associated with radiosensitivity [51]. Furthermore, the p53 status determined the cellular response to chemotherapy in an anticancer drug–dependent manner. Colon cancer cells with the *p53* gene deleted was found to be more sensitive to the DNA-damaging agent, doxorubicin, but was much more resistant to the antimetabolite 5-fluorouracil [52]. Taken together, these data indicate that because of the nature of tumor heterogeneity, the cellular response to chemoradiation is not solely determined by wt p53. However, understanding such responses in conjunction with p53 status would help the rational design of anticancer therapies to maximize their efficacy.

Gene therapy

To reintroduce wild-type p53. Because p53 function is lost in many cancers, it is logical to restore p53 function by reintroducing wt p53. One common gene therapy approach is the use of viruses to deliver p53. An early study, using retrovirus-mediated gene transfer of wt p53 into human lung cancer cells, showed the inhibition of tumor growth both *in vitro* and *in vivo* [53]. Gene therapy using human wt p53, delivered by replication-defective adenovirus (Ad-p53) for better transduction efficiency and lower toxicity, has been extensively studied in the preclinical and clinical settings with an impressive anticancer activity, resulting from p53-induced growth arrest and apoptosis [54]. The Ad-p53, under the brand name of Gendicine, or Advexin, has been currently in clinical use in China since 2003 [55] or in phase 1 to 3 clinical trial in the United States, respectively [7]. The results showed that Gendicine/Advexin is well tolerated in patients and efficacious in the treatment of numerous cancers, particularly head and neck cancer and lung cancer, as a single agent or in combination with chemotherapy or radiation therapy [7,56,57].

To eliminate mutant p53–containing cancer cells by adenovirus. Another p53-related gene therapy is the use of an E1B-deleted adenovirus, designated as ONYX-015, which selectively replicates in p53-deficient cancer cells and subsequently lyse the cells [58]. Preclinical studies showed that ONYX-015 has antitumor activity both *in vitro* and *in vivo*, particularly in combination with chemotherapy or radiation therapy [59,60]. Clinical trials revealed that ONYX-015 had a marginal antitumor activity when administrated alone, but a significant effect when combined with standard chemotherapies in a number of human cancers [56,61,62].

Small molecules. Reactivation of p53 and induction of tumor cell apoptosis (RITA) was identified through a cell proliferation assay using a pair of isogenic cancer cell lines differing in p53 status [63]. Biochemically, RITA bound to p53 at the N-terminal domain with a high affinity. Although an initial study showed that RITA could block p53-Mdm2 binding [63], a subsequent *in vitro* study using nuclear magnetic resonance suggested that it might not disrupt p53-Mdm2 binding [64]. Biologically, RITA suppressed tumor cell growth both *in vitro* and *in vivo* by inducing massive apoptosis in a p53-dependent manner in multiple cancer cell models [63]. A recent mechanistic study revealed that RITA, through activating p53, abrogates key oncogenic pathways. Specifically, RITA-activated p53 causes the transcriptional repression of antiapoptotic proteins, including Mcl-1, Bcl-2, survivin, and MAP4, downregulates oncogenic proteins, c-Myc, cyclin E, and β-catenin, and blocks the AKT pathway at multiple levels [65]. Thus, RITA can be further developed as a single agent or used in combination with chemoradiation for effective cancer therapy through p53-mediated abrogation of cancer cell survival and oncogenic pathways.

Targeting wt p53 — To Inhibit

Chemotherapy or radiation therapy kills cancer cells, but at the same time causes many adverse effects because of normal cell toxicity, resulting at least in part from p53 activation and apoptosis induction in normal proliferating cells/tissues, such as bone marrow, lymphoid organs, hair follicles, and epithelium lining of the small intestine. Temporary blockage of p53 activation in normal cells during the treatment of p53-deficient tumors should reduce these adverse effects [66]. Because p53 induces apoptosis through transcription-dependent and transcription-independent, but mitochondria-dependent mechanisms [20], two classes of small molecules have been identified, which target either p53 transcriptional activity or p53-mitochondrial binding activity, respectively. The first class of compound, designated as Pifithrin (PFT)-α, reversibly inhibits p53-dependent transcriptional activation and apoptosis and protects mice from the lethal dose of IR without promoting tumor formation [67]. The second class of compound, designated as PFT-μ, inhibits p53 binding to mitochondria by reducing p53-binding affinity to Bcl-xL and Bcl-2 without affecting p53 transactivation. Similar to PFT-α, PFT-μ also protects primary mouse thymocytes from p53-mediated

apoptosis on exposure to radiation and protects mice from radiation-induced lethal hematopoietic syndrome [68]. Thus, these two classes of compounds could be further developed for clinical use in combination of chemoradiation during the treatment of cancers, particularly those with p53 mutation or deletion for normal cell protection.

Targeting Mutant p53 – To Rescue

Because p53 mutation occurs in ~50% of human cancers, an effective cancer therapy would be the reactivation of p53 mutants. The rescue strategies vary dependent on the mutation types. In tumors harboring a DNA-contact mutant, the attempts were to introduce the functional groups that create new contacts or to stabilize the scaffolding positioning in the remaining contact sites [69,70]. The functions of conformation mutants can be restored by specific small peptides or small molecules that aid the proper folding of the unfolded p53 conformation [70].

Synthetic peptides

CDB3. CDB3 is a short synthetic nine-residue peptide (REDEDEIEW), derived from p53-binding protein 2 (53BP2 or ASPP), a known p53-binding protein that interacts with the p53 core domain and upregulates p53-dependent transactivation [71]. It was identified through a rational approach searching for small molecules that bind to p53 core domain and stabilize p53 [72]. The notion behind this approach is that a peptide binding with higher affinity to a properly folded state than to an unfolded form of the mutant will shift the equilibrium toward the active wild-type conformation. The *in vitro* studies showed that CDB3 indeed restored the sequence-specific DNA binding to various p53 mutants by stabilizing them in a bioactive conformation [72]. CDB3 also restored the transcriptional activity of two hot spot p53 mutants, R273H and R175H, for transactivation of two p53 target genes, *Mdm2*, *p21* [73]. Furthermore, CDB3 induced the accumulation of wt p53 and sensitized cancer cells to radiation [73]. Although as a small peptide, CDB3 will likely have poor bioavailability, it can serve as a lead for the further development of p53-reactivating small molecules.

p53 C-terminal peptide. In addition to CDB3, several other studies demonstrated that short synthetic peptides derived from the p53 C-terminal region (peptide 46) can reactivate mutant p53 through stabilization of the core domain folding and/or establishment of novel DNA contacts [74,75]. Although the exact mechanism of action is still unclear, these peptides were indeed shown to rescue the function of endogenous mutant p53 proteins, leading to growth inhibition, apoptosis, and suppression of solid tumor growth in an *in vivo* animal tumor model [76].

Small molecules

CP-31398. CP-31398 was the first small molecule that has activity to change p53 conformation from mutant to wild type. It was identified through a chemical library screening based on an *in vitro* biochemical assay that detects wt or mutant p53 conformation through two specific antibodies [77]. CP-31398 was found to stabilize the core domain and enhance transcriptional activity of p53 in living cells [77], but the detailed mechanism of action of CP-31398 remains elusive. Nuclear magnetic resonance studies failed to detect any binding of CP-31398 to the p53 core domain [78]. CP-31398 had no effect on p53-Mdm2 binding, did not cause DNA damage response, or induce p53 phosphorylation at Ser 15 or 20, but rather it increased wt p53 levels by reducing p53 ubiquitination [79]. Biologically, CP-31398 induced growth arrest and apoptosis in a number of human cancer cell lines both *in vitro* and *in vivo* [77,80] and inhibited UVB-induced skin carcinogenesis [81]. Furthermore, CP-31398 seems to have other targets in addition to p53 because it could induce cell death in both p53-dependent and -independent manners [70].

PRIMA-1 and MIRA-1. p53 reactivation and induction of massive apoptosis (PRIMA-1) and mutant p53 reactivation and induction of rapid apoptosis (MIRA-1) are two classes of compounds with unique chemical structures that were identified through a cell-based screening for compounds that suppress the growth of tumor cells expressing an exogenous mutant p53 [82,83]. The compounds were found to restore the sequence-specific DNA binding and change the mutant p53 conformation to wild type, leading to transactivation of p53 target genes [82,83]. In contrast to both CP-31398 and CDB3, PRIMA-1 does not activate wild-type p53. A recent mechanistic study revealed that PRIMA-1 is converted to a decomposition product that forms covalent thiol adducts on mutant p53 to restore its tumor suppressor activity [84]. Biologically, both compounds showed antitumor activity in multiple cancer cell lines and *in vivo* xenograft tumors harboring a mutant p53 without apparent normal tissue toxicity [82–84]. Furthermore, PRIMA-1 was found to be active against p53-null cancer cell lines through a mechanism involving the JNK pathway and heat shock protein 90 [85,86]. Finally, PRIMA-1 or its analog, PRIMA-1(met), sensitized lung cancer cells or prostate cancer cells to adriamycin or radiation, respectively [87,88] and inhibited *in vivo* xenograft tumor growth [89].

Ellipticine. Ellipticine and its derivatives were initially identified in the drug sensitivity profiling of the NCI-60 cancer cell line panel to be more effective against tumor cells with a mutant p53 [90]. An ellipticine derivative, 9-hydroxyellipticine was found to increase the transcription of p21 and Bax and to induce G_1 arrest in a mutant p53-dependent manner [91]. The detailed follow-up work showed that ellipticine changed the p53 conformation from mutant to wild type, restored the sequence-specific DNA binding and transactivation of p53-driven luciferase reporter, and activated mutant p53 to induce p53 target genes, *p21* and *Mdm2* in mouse xenograft tumor tissues [92]. Furthermore, ellipticine was recently identified to increase nuclear localization of both wt and mutant p53 in a manner independent of DNA damage [93]. Because the ellipticine series of compounds has many off-target activities including promoting p27 degradation [94], it is unlikely to be further developed for the clinic use.

WR-1065. WR-1065 (aminothiol) is an active metabolite of the cytoprotector amifostine and acts as a classic scavenger of reactive oxygen species [95]. WR-1065 was found to activate both wt and mutant p53 and increase the expression of p53 target genes in a manner independent of DNA damage [96,97].

P53R3. p53R3 is a recently identified small molecule that restores the sequence-specific DNA binding of p53 mutants (R175H and R273H) after screening a small library of compounds using an *in vitro* gel shift assay [98]. The compound was found to enhance the recruitment of both wt and mutant p53 to target promoters and to induce the expression of a number of p53 target genes. Biologically, the compound induces mutant p53-dependent growth arrest and sensitizes TRAIL-induced cell death in multiple glioma cell lines [98].

Targeting Mutant p53 – To Kill

Synthetic lethality for p53 mutation. Synthetic lethality is a situation where a cancer-associated mutation itself is nonlethal but renders cancer cells susceptible to a second hit that becomes lethal on inactivation [99,100]. In the case of p53, which is mutated in 50% of human cancer, application of a synthetic lethality strategy to identify chemical compounds that selectively kill human cancer cells harboring a mutant p53 will be of significant importance for the discovery of a novel class of anticancer drugs (Figure 2). Synthetic lethality has been shown in a number of human cancer cells with deletion of PTEN or DPC4, mutation of K-Ras or B-Raf, and overexpression of c-Myc [101–105], demonstrating the feasibility of using synthetic lethality strategy for anticancer drug discovery. The p53 synthetic lethal drugs, if identified and developed, can be used 1) for cancer treatment to selectively kill mutant p53-containing cancer cells and 2) for chemoprevention to eliminate mutant p53-containing cancer-prone cells, at the early stage of carcinogenesis. Furthermore, p53 synthetic lethal drugs should have, in theory, minimal adverse effects, because normal cells do not contain a p53 mutation.

A "synthetic lethal-like" screening was conducted in a panel of 60 NCI cancer cell lines in an attempt to identify compounds that selectively inhibited the growth of cancer cells devoid of p53 [106]. Few classes of compounds were identified, and the only "popular" hit was paclitaxel, an inhibitor of microtubule polymerization [106]. It was speculated that microtubule-associated protein 4, a p53 transcriptional repressed target, may mediate the sensitivity of cells lacking p53 activity to paclitaxel-induced cell killing [107]. Another drug that selectively inhibited p53-deficient tumor cell growth is metformin, a diabetic drug, with the mechanism associated with activation of AMP kinase and inhibition of oxidative phosphorylation, which created an environment more vulnerable to mutant p53 cells [108].

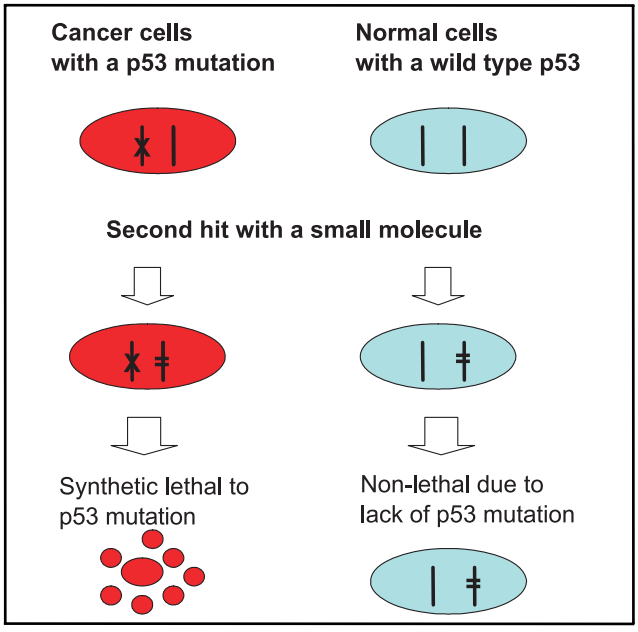


Figure 2. Synthetic lethality for p53 mutation: Synthetic lethality refers to the situation in which the cancer-associated mutation itself is nonlethal but renders cancer cells susceptible to the second hit, which results in lethal phenotype. Because p53 is most frequently mutated in more than half of human cancer cells, it is feasible in theory to use this strategy to identify drug candidates that preferentially kill cancer cells with a p53 mutation. The p53 synthetic lethal drugs, if identified and developed, should have a minimal toxicity to normal cells and can be used for cancer chemoprevention and treatment of mutant p53–containing cancers.

Small molecules that abrogate the G_2/M checkpoint control can be considered to act through the synthetic lethal mechanism against p53 mutation. The hypothesis is based on the fact that p53-deficient cells have abrogated G_1 checkpoint control (lack of p53-mediated p21 induction in response to DNA damage), and thus, further abrogation of G_2 checkpoint control will selectively kill p53-deficient cancer cells through the induction of mitotic catastrophe. This turns out to be the case. UCN01, a potent G_2 checkpoint abrogator sensitized p53 mutant cancer cells to IR by abrogating G_2 checkpoint control, whereas cancer cells with wt p53 were much more resistant [109]. Similarly, our previous work showed that PD0166285, a potent Wee-1 kinase inhibitor that abrogated G_2 checkpoint control, selectively sensitized p53 mutant cancer cells to radiation [110]. A recent study from Vogelstein's group further supported this notion of synthetic lethality. Transcription profiles of four paired colon cancer cell lines, isogenic for p53 deletion or mutation, revealed a consistent up-regulation of polo-like kinase 1 (PLK-1), a well-known protein that regulates G_2/M checkpoint control, in p53-deficient lines after exposure to IR. Consistently, p53-deficient cells are much more sensitive to PLK-1 inhibitors when combined with p53 activators, such as radiation and Nutlin-3. Furthermore, *in vivo* xenograft tumor studies showed that a PLK-1 inhibitor used as a single agent caused significant regression of p53-null tumors [111]. These studies highlight the feasibility of using synthetic lethal mechanism for effective cancer therapy.

We have recently attempted to identify novel small molecules that act through synthetic lethal mechanism to selectively kill cancer cells with a mutant p53 mutation. We conducted this p53 synthetic lethal screening using a well-characterized p53 temperature-sensitive model in which p53-null H1299 cells were transfected with a temperature-sensitive mutant p53A138V (H1299-p53^{ts}). The p53 in these cells adopts a mutant conformation when grown at 39°C and a wild type conformation at 32°C. Temperature shifting from 39 to 32°C restores the wild type p53 conformation that induces growth arrest but not apoptosis [112,113]. Screening of both chemical and natural product libraries was conducted at both temperatures, and the compounds that selectively killed cells at 39°C (mutant p53 status), but not at 32°C (wt p53 status), were subjected to the secondary counterscreening. In the counterscreening, we used H1299-neo vector control cells at both temperatures to filter out potential false-positives derived merely from temperature shifting. Candidates that showed selective killing of H1299-p53^{ts} at 39°C only were identified, and they are being further characterized (unpublished data). A disadvantage of this type of screening is the lack of mechanism of drug action, which will need follow-up study to identify the "second" target that is synthetically lethal to p53 mutation. The ideal situation would be to conduct simultaneously the same screening using a small interfering RNA (siRNA) library, leading to identification of the "second" target.

Elimination of cancer cells with the gain-of-function p53 mutants. During the validation of our candidate hits, identified from this p53 synthetic lethal screening, we serendipitously found that cardiac glycoside drugs, such as digoxin and ouabain, reduced the p53 levels in a time- and dose-dependent manner in a subset of cancer cell lines but not in immortalized normal cell lines. The drug sensitivity to

p53 reduction is cancer cell line–dependent but independent of p53 status of a wild type or mutants. A mechanistic study revealed that the drug-induced p53 decrease neither occurs at the messenger RNA level nor is due to enhanced degradation, which is a common mechanism for p53 regulation. Rather, it occurs at the protein level as a result of reduced *de novo* synthesis of p53 protein. The drug-induced p53 reduction can be rescued by the inhibitors of Src and MEK, suggesting an involvement of Src/mitogen-activated protein kinase signaling pathways, initiated on the drug binding to Na^+/K^+-ATPase [114]. Given the fact that glycoside drugs are being used in clinic for the treatment of congestive heart failure [115] and that cardiac glycosides are inactive against wt p53 in normal cells, but potently active in the elimination of mutant p53 in some cancer cells, the drugs may have utility in the treatment of human cancers harboring a gain-of-function p53 mutant [116,117].

Targeting p53 Regulators – To Activate p53

Mdm-2 (Murine Double Minute-2, Hdm2 in Human)

Another effective approach to activate wt p53 is to inhibit its negative regulators. The best-known naturally occurring p53 inhibitor is its downstream target, Mdm2 [118]. Mdm2 encodes a 90-kDa protein that was first identified as the gene responsible for the spontaneous transformation of immortalized murine BALB/c 3T3 cells [119]. It contains a p53 binding domain at the N-terminus and a RING domain at the C-terminus. Mdm2 inhibits p53 through two well-characterized mechanisms: 1) Mdm2 binds to p53 at the N-terminus and blocks p53 transactivation activity and 2) Mdm2 acts as an E3 ubiquitin ligase to promote p53 degradation. In addition, Mdm2 also exports p53 out of nucleus where p53 acts as a transcription factor [34,120]. Mdm2 is overexpressed in approximately 7% of human cancers with a much higher incidence in soft tissue tumors, such as osteosarcoma [121]. Both *in vitro* and *in vivo* studies indicated that oncogenic activity of Mdm2 is mainly attributable to its binding and degrading p53 [122,123]. Thus, inhibition of Mdm2 in this subset of human cancer should reactivate p53 to induce cell killing. Indeed, this notion was supported by many proof-of-concept studies, including 1) the blockage of the interaction between Mdm2 and p53 with synthetic peptides or monoclonal antibodies [124] and 2) reduction of Mdm2 levels with antisense oligonucleotides or siRNA [125]. So far, two classes of small molecules have been identified, which either disrupt Mdm2-p53 binding or inhibit Mdm2 E3 ubiquitin ligase activity to reactivate wt p53.

Small Molecules that Disrupt Mdm2-p53 Interaction

Historically, it has been difficult to develop small-molecule inhibitors that disrupt large protein-protein interactions. However, the crystal structure of the Mdm2-p53 peptide binding revealed that the binding relies on the contact of the p53 peptide side chains of Phe19, Trp23, and Leu26 with the N-terminus of Mdm2 (amino acids 17-125) in a deep hydrophobic pocket [126], which made it possible for small molecules to disrupt binding. Several classes of structurally distinctive compounds have been reported to disrupt Mdm2-p53 binding [127,128]. These compounds include Nutlins, benzodiazepinediones (BDAs), and an Mdm2 inhibitor (MI) series of spiro-oxindoles derivatives, including MI-63, MI-219, and MI-43 [127,129–133].

Nutlins. The nutlin series was the first class of small molecules, identified through a screening of a diverse library that disrupted Mdm2-p53 peptide binding. This series of compounds effectively abrogates Mdm2-p53 interaction through a high-affinity binding to Mdm2 [129] with a high selectivity for Mdm2 over Mdmx, a homologue of Mdm2 [127]. Nutlin-3, an analog of the series, induced p53 levels, activated p53 transcription activity, and has a broad activity against cancer cells harboring a wt p53 both *in vitro* and *in vivo* [129]. Examples include colon and breast cancer cell lines and osteosarcoma cells [129], lymphoblastic leukemia [134], and retinoblastoma [135]. In combination with chemoradiation, Nutlin-3 showed synergistic activity against prostate cancer [136], lung cancer [137], lymphocytic leukemia [138,139], and neuroblastoma [140]. Nutlin-3 also showed some normal cell protective activity against chemotherapy by inducing cell cycle arrest [141,142]. Furthermore, Nutlins showed a direct antiangiogenic and antiosteoclastic activity, which may have an application for tumors harboring a mutant p53 [143].

Benzodiazepinedione. Benzodiazepinedione (BDA) and its derivatives were identified through a chemical library screening using a miniaturized thermal denaturation-based assay. X-ray crystal structure confirmed that the BDA interacts with the p53-binding pocket of Mdm2 [130]. The compounds increased the p53 transcriptional activity, inhibited the proliferation of cancer cells in a wt p53–dependent manner, and synergized with doxorubicin to inhibit tumor cell growth both *in vitro* and *in vivo* [130,133].

MI series. The MI series of spiro-oxindole compounds, including MI-219, MI-63, and MI-43, were discovered through structure-based design by Ding et al. [131] by mimicking all four Mdm2-contacting residues (Phe19, Trp 23, Leu 22, and Leu 26) on p53. The MI series of compounds, with MI-219 being the most potent, bind to Mdm2 with a high affinity. The drug-induced disruption of Mdm2-p53 binding caused p53 accumulation leading to up-regulation of many p53 target genes and to induction of apoptosis in a number of human cancer cell lines derived from breast, colon and prostate cancers *in vitro* in a wt p53–dependent manner. MI-219 as a single agent also caused a complete inhibition of xenograft tumor growth *in vivo* at a dose that has no obvious toxicity to animals [127,144,145].

We tested the efficacy of MI-43 against human lung cancer cells and found that the compound preferentially inhibited the growth of wt p53-containing cells over p53-null cells. MI-43 induced the growth arrest at both G_1 and G_2 phases of the cell cycle, at the low concentration as a result of p21 induction and apoptosis at the high concentration due to Puma/Noxa induction. Importantly, MI-43 was much less toxic to normal lung cells than cancer cells. Finally, when used in combination, MI-43 sensitized chemoresistant A549 cells to etoposide-induced apoptosis [132].

Mdm2 E3 ubiquitin ligase inhibitors

HLI98. The HLI98 series of compounds was identified through the high-throughput screening of a chemical library using an *in vitro* Mdm2 autoubiquitination assay [146]. The follow-up experiment showed that HLI98C, an analog, indeed inhibited Mdm2 E3 ligase activity. In the cell-based assays, the compound stabilized p53 and Mdm2 and activated p53-dependent transcription and apoptosis. The compound was much more efficacious against cancer cells with wt p53, although it demonstrated some p53-independent cytotoxicity [146]. Although the evidence of *in vivo* antitumor activity for HLI98C was lacking [146], this proof-of-concept study did indicate that an Mdm2 E3 ligase inhibitor, which has selective activity against wt p53–containing cancer cells, can be identified.

Two concerns should be borne in mind in the clinical development of Mdm2-based inhibitors for anticancer therapy. The first is the therapeutic window because the compounds would also induce p53 in normal cells to cause some adverse effects. Second is the Mdm2 itself, which is accumulated after compound treatment as a result of p53 activation. In addition to targeting p53, Mdm2 interacts with a number of other proteins, such as p73, p63, E2F1, and HIF1α [147–149]. A recent study also showed that Mdm2 caused the accumulation of XIAP to inhibit apoptosis [150]. These p53-independent but Mdm2-dependent effects would eventually affect the efficacy of this class of compounds.

SirT1/SirT2

SirT1 and SirT2 are two members of the NAD^+-dependent class III histone deacetylases with a total of seven members in humans [151,152]. SirT1 and SirT2 catalyze the reaction between an acetylated lysine with NAD^+, leading to the production of deacetylated lysine, 2′-*O*-acetyl-ADP-ribose, and nicotinamide [153]. It is well established that acetylation of p53 at lysine 382 enhances p53 DNA binding activity [154]. Thus, SirT1-catalyzed deacetylation of p53 at lysine 382 would deactivate and destabilize p53 [155–157]. These studies established that SirT1 is yet another negative regulator of p53.

Tenovin-1 and Tenovin-6. This series of compounds was identified in a cell-based screening for compounds that activate p53, using a p53-driven transactivation assay [158]. Tenovins stabilized wt p53, induced p53-dependent cell cycle arrest and apoptosis, and suppressed xenograft tumor growth *in vivo* as a single agent [158]. Mechanistically, Tenovins are nongenotoxic agents and do not induce DNA damage. Rather, they inhibited NAD-dependent deacetylase SirT1/T2, thus restabilizing and reactivating p53. Tenovins, therefore, are a novel class of p53-activating agents that can be further developed for clinical use as well as for the study of sirtuin function as biological tools.

JJ78:12. This series of compounds was also identified by Lain et al. [158] in the same reporter assay used for p53 activator screening. The JJ78:12 series contains much more potent p53 activators with a nanomolar dose range and showed clear anticancer activity both *in vitro* and *in vivo*. Mechanistically, this series of compounds caused tubulin depolymerization and may not be further developed for clinical use because of their high cytotoxicity, resulting from tubulin poison [159]. Interestingly, our effort in screening for compounds that change p53 conformation from mutant to wt using a luciferase reporter–based H1299-p53 temperature-sensitive model [112,113] led to the identification of a series of microtubulin poison compounds, including nocodazole, albendazole, parbendazole, and mebendazole (unpublished data), suggesting a nonspecific or indirect nature of p53 activation by this class of compounds.

Targeting Other p53 Family Members

p53 has two family members, p73 and p63 with significant sequence homology among them [160,161]. Like p53, both p73 and p63 bind to the p53-specific DNA binding motif, transactivate p53 downstream target genes, and suppress tumor cell growth by inducing growth arrest and apoptosis [160,161]. The tumor suppression activity of p73 and p63 was further demonstrated in a mouse knockout study, which showed various tumor predispositions on heterozygous deletion of either gene [162]. Unlike p53, p73 or p63 is rarely mutated in human cancers [163] but could be inactivated by binding to a subset of p53 mutants [164]. Targeting p73 and p63 has been proposed for cancer therapy [165,166] with approaches including gene therapy, small peptides, and small molecules.

Gene Therapy

Like the gene therapy approach using wt p53, the same approach has been extended to its family members, p73 and p63. The adenovirus-mediated transduction of p63 and p73 into tumor cells was found to be an alternatively efficient gene therapy approach both *in vivo* and *in vitro* [167,168], especially when tumors are resistant to p53-mediated gene therapy [168,169]. Ad-p73 demonstrated a significant cytotoxicity against multiple cancer cell lines tested by inducing both growth arrest and apoptosis [169]. Ad-p73 also sensitized p53 mutant–containing cells to adriamycin with a higher efficiency than Ad-p53 [169]. The apoptosis-inducing effects of Ad-p63γ were also found to be greater than those of wild-type p53 in osteosarcoma cells with Mdm2 amplification [168].

Small Peptides

Short-interfering mutant p53 peptides. One activity of gain-of-function p53 mutants is to physically interact with, sequester, and inactivate p73 [170,171]. Short-interfering mutant p53 (SIMP) peptides were designed based on mutant p53/p73 binding regions. Indeed, SIMP effectively disrupted the protein complex of mutant p53/p73 and restored the p73 function. Biologically, SIMP sensitized mutant p53-containing tumor cells to adriamycin and cisplatin. Notably, the effects of SIMPs are mutant p53-specific and had no effect on cancer cells either with a wt p53 or with a p53-null [172].

37AA. This peptide consists of 37 amino acids from p53 after a noncontiguous fusion of evolutionarily conserved boxes II and III on the DNA binding domain of p53. The 37AA induced cell death through activation of p53/p73 target genes in p53-null cells. Further studies revealed that 37AA was able to bind to iASPP, an inhibitory member of the ASPP family, resulting in the release TA-p73 (a p73 isoform) from iASSP/TA-p73 complex. Anticancer activity of 37AA was further demonstrated in a colon cancer xenograft model, in which 37AA was driven by an expression vector and delivered in dendrimer-based nanoparticles [165,173].

Small Molecules

Wang et al. [174] recently identified a number of small molecules in a screening for activating p53 target genes and apoptosis in p53 mutant–containing cells. The follow-up studies using an siRNA-silencing approach confirmed that some of these compounds mediated their activity through transactivating p73. Although the detailed mechanism is unknown, these compounds had anticancer activity when assayed *in vivo* using the p53-null HCT116 xenograft tumor model [174].

Reactivate transcriptional activity. Reactivate transcriptional activity (RETRA) was identified in a cell-based screening for compounds that reactivate the transcriptional activity of p53 in mutant p53–containing cells [175]. Follow-up studies showed that RETRA activates a number of p53 target genes and selectively inhibited the growth of mutant p53-containing cancer cells both *in vitro* and in mouse xenografts. Mechanistic studies in mutant p53–containing cells revealed that the compound increased the p73 levels and released p73 from complexing with mutant p53. Importantly, RETRA is active against mutant p53–containing

cancer cells without affecting normal cells. Taken together, these studies demonstrated that reactivation of p73 from p53-null or p53 mutant cells is a promising approach for effective cancer therapy.

Conclusions and Future Perspectives

Cancer is a complex disease with multiple genetic and epigenetic alterations. Genetic alterations in any given cancer, even those originating from the same tissue/organ, could have a dramatic difference. Conversely, cancers derived from different tissues/organs may have similar alterations in a given signaling pathway. Thus, an effective personalized cancer therapy requires a thorough understanding of genetic and epigenetic alterations of each individual cancer, followed by rational design of combinational therapies targeting these altered molecules and pathways. These drugs, if further developed from p53 lead compounds described in this review (Table 1), will revolutionize the current cancer therapies by targeting p53 and its regulators on an individual tumor basis. For example, for wt p53–containing tumors, chemoradiation can be used in combination with p53-activating drugs. Similarly, chemoradiation in combination with p53-reactivating drugs or drugs acting through a synthetic lethal mechanism could increase the efficacy against tumors with a mutant p53. The synergistic effect on cancer cell killing of p53 drugs–chemoradiation combination allows a lower dose regimen of routine chemoradiation to reduce normal cell/tissue toxicity. Another approach to reduce normal cell/tissue toxicity is to use p53 inhibitors on a temporary basis during chemoradiation therapy. Furthermore, p53 drugs can be used in combination with other to-be-developed mechanism-driven drugs to achieve a synergistic effect by targeting the same signaling pathway both upstream and downstream. One example will be the combination of Nutlins or MI-219, which activates p53, but with an adverse effect of Mdm2 accumulation [127,132,144], with Smac mimetic drugs, which disrupt XIAP-caspases inhibitory binding to release activated caspases [176–178]. A recent report showed that Mdm2 causes the accumulation of XIAP [150]. This mechanism-driven combination would lead to apoptosis induced by p53 being fully executed with activated caspases. Furthermore, as we gain a better understanding of p53 signaling pathways, additional p53-related targets, upstream and downstream of p53, can be identified and validated for future discovery of novel compounds that target p53 signaling pathways [26,179].

Finally, as the old proverb goes, "prevention is the best medicine." Chemoprevention is a widely accepted concept, aiming to kill cancer-prone cells at the early stage of carcinogenesis to prevent tumor formation in the first place. Nontoxic natural products from vegetables, teas, and fruits as chemoprevention agents have been extensively studied, and some promising components have been advanced to clinical trials [180–183]. Although our current effort in discovery of p53 drugs is mainly focused on cancer therapy, one promising future direction will be to identify and fully develop natural products, that act through a synthetic lethal mechanism to kill cancer-prone cells with p53 mutations, as chemoprevention drugs. Thus, p53 provides us many opportunities in our fight against cancer, a deadly disease to humankind.

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