An important function of the p53 tumor suppressor is to induce cell cycle arrest or apoptosis of tumor cells in response to DNA damage, oncogene activation, and hypoxia (1–4). This function is dependent, in part, on the sequence-specific DNA-binding of p53 and subsequent transcriptional activation of target genes that mediate the biological effects of p53. Activation of p53 by cytotoxic therapeutic agents has been shown to result in apoptosis that is relevant for therapeutic response, both in animal models and in the clinic. Activation of p53 by radiation or chemotherapeutic agents also contributes to the toxic effects of therapy. Importantly, in just over 50% of all tumors, the DNA-binding domain of p53 is mutated (5). These single amino acid substitutions target residues that contact DNA directly (Fig. 1), as well as residues that stabilize the scaffold that orients the structure of the DNA-binding interface (6). Mutations in p53 that result in loss-of-function of the wild-type activities of p53 can also lead to gain-of-function properties (7). Some mutant p53 proteins gain transforming activities that are due to differential binding to specific promoter sequences (8). Because p53 functions as a tetramer in cells, mutant p53 proteins can act in a dominant-negative fashion to inhibit wild-type p53 function (9).

In principle, the functional activity of p53 mutants could be restored either by introducing novel p53-DNA contacts (Fig. 1), or possibly by promoting the stability of the folded state of p53 even in the absence of certain DNA contacts residues (10).

Methods used to restore wild-type p53 function to mutated p53 include the introduction of second-site suppressor mutations, as well as the identification of small molecules or peptides (e.g., CP-31398, PRIMA-1, and ellipticine), which stabilize the active p53 conformation in cells (11). Some strategies have been developed that rely on small peptides, for example, those derived from the COOH-terminal region of p53 or the p53 binding domain of the p53BP2 protein (CBD3), to modulate DNA-binding and transcriptional responses by mutant p53 (12).

In the many tumors that retain a wild-type p53 gene sequence, the p53-dependent pathways for cell-cycle arrest and apoptosis are sometimes deficient, due to overexpression of mouse double minute 2 (MDM2) or deficiency in the alternate reading frame protein at the INK4a locus (13). As such, some efforts have been directed at the systemic activation of wild-type p53 in tumor cells that retain wild-type p53 through the use of small molecules such as the Nutlin3 (14) or through use of peptides that target MDM2 (Fig. 2). Another approach for activating endogenous wild-type p53 (as well as mutant p53) involves modulation of COOH-terminal autoregulation of p53. Specifically, strategies to activate wild-type p53 have included the use of synthetic peptides derived from the p53 terminus (15–17), antibodies that target the COOH terminus (18), and acetylation of the COOH terminus (19).

Radiation and chemotherapeutic agents induce p53-dependenent apoptosis in cells, and because the p53 gene is expressed in normal tissues, most if not all of the proliferating normal tissues are also prone to apoptosis. With recent evidence that the acute pathologic response to DNA damage makes no measurable contribution to p53-mediated tumor suppression in mice (20), the idea of reversibly inhibiting p53 activity to avoid the negative side effects of therapy has begun to gain importance for modulation of the therapeutic window. Pifithrin is a small molecule that has been shown to reversibly inhibit p53 activity in vivo and shows potential for reducing the side effects of cancer therapy (21).
Recent efforts directed at functional screening for small molecules that restore p53 transcriptional responses in p53-deficient cells (Fig. 2) suggest that the p53 family, such as p73, can be an important therapeutic target (22). Specifically, small molecules may increase expression of p73 and restore proapoptotic effects normally observed downstream of wild-type p53 activation by classic therapeutic agents. Functional screens, subsequent validation experiments, and in vivo studies demonstrating antitumor effects may provide a means to identify and further develop small molecule therapeutics that target mutant p53 to restore wild-type p53 responses (Fig. 2). Restoration of wild-type p53 function to mutant p53 proteins has been a holy grail in cancer drug development, yet the identification of small molecules that directly effect mutant p53 has remained elusive. Nonetheless, it is important to continue rational drug design to modulate p53 function as pharmacologic intervention in that the activity of both mutant and wild-type p53 could have major effect on the efficiency of chemotherapy and radiation treatments.

In this review, we will detail strategies that have been developed to modulate the structure or function of p53 for the purpose of drug development. We will mention key results that provide insights into potential future directions, as well as discuss each of the areas in terms of limitations. It is clear that multidisciplinary efforts are required to continue to address the therapeutic modulation of p53, and thus, it is our goal to provide a review that can summarize the state of the field, as well as serve as a starting point for such continued efforts.

The Amino- and Carboxy-Termini of p53 Are Involved in Allosteric Regulation of the DNA-Binding Domain

In response to stress signals, wild-type p53 inhibits tumor cell growth by inducing cell cycle arrest, apoptosis, senescence, or differentiation, depending on cell type and environment (23). p53 also has roles in DNA damage repair, angiogenesis antagonism, cytokine production, and melanin synthesis (24). p53 activity must be suppressed in normal cells to allow for normal cell division, and rapidly induced in response to certain cell stresses. In cells, p53 activity is controlled by regulation of p53 protein levels and the subcellular localization of the protein, as well as through regulation of its transcription factor.
Recent evidence suggests that specific p53-interacting proteins can regulate the selectivity of p53 target gene activation (25, 26).

The structure of p53 consists of three major domains, including the amino-terminal transactivation domain, the core DNA-binding domain, and the carboxy-terminal tetramerization domain (6). The vast majority of tumor-derived p53 mutations lead to amino acid substitutions in the DNA-binding domain, although mutations in the carboxy terminus domain have been reported (Fig. 1). Interactions between the COOH terminus with the core domain has been proposed to allow for allosteric regulation of p53 activity. The negative regulator MDM2 binds and sequesters the NH₂-terminal transactivation domain of p53 in a negative feedback loop (27). MDM2 interaction with the amino-terminus as well as with the DNA-binding domain can also lead to changes in p53 conformation as well as to p53 degradation through the ubiquitin-mediated proteasomal pathway (28). The MDM2-p53 interaction is inhibited by p14ARF and/or the phosphorylation of p53 and MDM2 by checkpoint kinases (23). MDMX, a homologue of MDM2, with ~50% sequence similarity in the p53-binding domain (29), negatively regulates p53 by inhibiting p53-dependent transcription (30). The amino acids in p53 necessary for binding MDM2 and MDMX are identical and located entirely in the transcription activation domain (31). DNA damage activates p53-dependent transcription through a signal transduction pathway involving all three p53 domains (32). The COOH terminus of p53 has been proposed to modulate its transcriptional activity by binding the core domain and inhibiting sequence-specific DNA-binding. In response to DNA damage, the NH₂ terminus is phosphorylated, which in turn leads to acetylation of the COOH terminus. The acetylated form of COOH terminus can no longer interact with the DNA-binding domain, resulting in activation of transcription. DNA damage–induced acetylation of p53 can also interfere with its ubiquitination at the same COOH-terminal lysine residues.

The p53 core domain, residues 102 to 292, is responsible for the sequence-specific DNA-binding activity of p53. The core domain consists of a β sandwich, a loop-sheet-helix motif, and loops L2 and L3 with 15 and 32 residues, respectively (6). The loop-sheet-helix contacts the major groove, the L3 contacts the minor groove, and the L2 loop stabilizes L3. The β sandwich has two antiparallel β sheets with four and five strands, and is important for stabilizing the DNA binding-site in the co-crystallized structure. The so-called hotspots for p53 mutation occur within the core domain, including at or near locations of p53-DNA contact. Mutations of DNA contacts sites, as expected, result in loss of sequence-specific DNA-binding and transactivation. The six hotspots for mutation include Arg248, Arg273, Arg175, Gly245, Arg249, and Arg282 (Fig. 1). Of these, only Arg248 in the L3 loop, Arg273 in the loop-sheet-helix, and Arg282 have been shown to act as DNA contact sites. The remaining hotspot mutations as well as the numerous other tumor-derived, non-hotspot mutations are postulated to provide a backbone scaffold that maintains the integrity of the DNA-binding pocket. Interestingly, of the six most common mutations, Gly245 is the only non-arginine. The glycine is critical because substitutions at this position could sterically hinder a conformation that would not be favored.

The oligomerization domain on the carboxy terminus of p53 allows it to form a tetramer, with each monomer binding to 1 pentamer within the 4 pentamer-containing consensus DNA-binding site (6). The consensus binding site for p53 consists of 2 copies of the 10-bp motif 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’ separated by 0 to 13 bp. Each copy of the monomer has internal symmetry and contains two oppositely oriented DNA-binding pockets.
pentamers with the sequence 5'-PuPuPuC(A/T)-3'. The dimer also contains this symmetry, with four pentamers positioned in alternating directions (33).

**Introducing Second-Site Suppressor Mutations that Create Novel p53-DNA Contacts or Stabilize the p53 Native Conformation can Restore Function to Mutant p53**

One approach to restoring wild-type function to p53 mutants is the identification of a second mutation that corrects the negative effect of the original mutation (Fig. 1). Halazonetis and colleagues used this approach, with the hypothesis that the introduction of novel p53-DNA contacts should be sufficient to rescue mutants in which sequence-specific DNA-binding activity has been lost (24). The seven most common tumor-derived mutants used in the study were as follows: p53His175, Gln248, Trp248, Ser249, His273, Trp282, and Cys273. Three of these mutations, classified as class I mutants, target arginines 248 or 273 that contact the DNA backbone (6). The four remaining mutations, called class II mutants, target arginines 175, 249, or 282 that stabilize the DNA-binding domain structure. A previous study indicated that some class I p53 mutants can bind DNA when allosterically activated by deletion of COOH-terminal regulatory domain or by monoclonal antibodies that mask the regulatory region. Knowing that all three of the chosen class I mutants bind DNA to some extent, the goal was to increase the affinity of these mutants for DNA by substituting residues in the DNA-binding domain with basic amino acids. Residues that are oriented close to the DNA backbone were chosen for substitution, whereas residues that already contact DNA or have an important role in scaffold stabilization were avoided. Of all the substitutions tested, only the Thr284 to Arg substitution showed enhanced DNA-binding affinity. The Thr284 to Arg substitution was found to enhance DNA-binding, transcriptional activity, and tumor suppressor function in class I mutants p53His273, p53Cys273, and p53Gln248, but in most cases, allosteric activation was still required. The Thr284 replacement did not affect the sequence-specificity of DNA binding in any of the mutants. It has been hypothesized that the Thr284 replacement creates an additional p53-DNA contact. The crystal structure indicates that Thr284 lies in the major groove of DNA but does not make extensive DNA backbone hydrogen bonds in DNA models. The V143A cancer mutant disrupts the L3 loop by inserting a serine residue in a very tight space that can only accommodate a small glycine. The N239Y suppression mutation most likely overcomes this L3 loop destabilization by introducing a DNA contact. The S240N suppression mutation also targets the L3 loop destabilization by introducing additional hydrogen bonds (10). The R249S cancer mutation most likely eliminates a guanidinium group that stabilizes the DNA-binding domain of p53. The L2 and L3 loops, and the H168R suppressor may overcome this by reintroducing a guanidinium group in the vicinity. The Fersht group also identified H168R as a suppressor mutation for R249S and showed that H168R restores wild-type conformation to loops 2 and 3 in R249S mutants (36). The molecular modeling in this study indicates that cancer mutations can be grouped into subsets based on their position within the DNA-binding domain. Because the suppressor mutation is located within the same domain as the original mutation, it may be possible to design a small molecule that stabilizes the DNA-binding domain of p53, and lower the free energy of the native wild-type conformation even in the presence of destabilizing mutations, and this may target an entire subset of cancer mutants.

**Small Molecules Restore Wild-Type p53 Activity to Cells Harboring Destabilized p53 Mutants by Stabilizing the Wild-Type p53 Conformation**

The restoration of wild-type conformation and activity to p53 mutants is a promising strategy for cancer therapy, due to the large frequency of p53 mutations in cancer. Different types of mutations may require different strategies for rescue. DNA-contact mutants require the introduction of functional groups that can create new contacts, as described previously in the suppressor mutation studies, or stabilize the scaffolding that positions the remaining DNA-contact sites. Unfolded mutants may be restored by specific small molecule or peptide agents that aid refolding or prevent the unfolding of the active conformation of newly synthesized p53. In principle, a small molecule that binds to properly folded wild-type p53, but not destabilized p53 with "mutant" conformations, will cause the equilibrium to shift toward the native state and allow for time-dependent accumulation of active wild-type p53 in cells (12). Several groups have used this approach in attempts to identify compounds that restore the conformation and activity of p53 mutants.
CP-31398. CP-31398 is a quinazoline-based small molecule that stabilizes the active conformation of p53 in cancer cells with mutant or wild-type p53 (37). It is the founding member of a group of compounds that stabilize the 1620 epitope located within the wild-type DNA-binding domain of p53 and blocks time- and temperature-dependent degradation of the 1620 epitope on a purified p53 core domain (37, 38). Additional acridine and phenothiazine-based active compounds that were identified share similar features such as a hydrophobic group joined to an ionizable group via a linker of specific length (6–8 Angstroms). CP-31398 was found to increase steady-state levels of the wild-type 1620 epitope by 5-fold in cells harboring mutant p53. This same result was also found in tumor xenografts in mice. In the presence of mutant p53, CP-31398 increased sequence-specific transcription activity of p53 by 10-fold and inhibited the growth of small human tumor xenografts up to 75% (37).

Despite the strong biochemical evidence that CP-31398 enhances the thermostability of purified p53 and the biological evidence that wild-type p53 activities are restored in cells with mutant p53, questions have lingered about evidence of a physical association between the compound and p53. Demma and colleagues (39) have shown that CP-31398, like the CDB3 peptide, which physically associates with p53, is able to restore the specific DNA-binding activity of mutant p53 proteins in a defined system. In a study that challenges the findings that CP-31398 acts directly on the p53 core domain, no interaction between the p53 core-domain and CP-31398 was detected in vitro using a wide range of quantitative biophysical techniques and a wide-range of experimental conditions (38). However, it should be noted that the soluble form of the in vitro prepared p53 DBD, unlike the native p53 form in cells, does not display the 1620 epitope.

CP-31398 and its derivatives have potential as anticancer drugs, but without a defined site of interaction on the DNA-binding domain, the precise structural mechanistic basis for the action of CP-31398 on mutant p53 protein remains a matter of speculation. Although CP-31398 activates mutant p53 in vitro and in cells, it does so without (38) blocking the interaction of p53 with MDM2 in vitro (40). CP-31398 clearly acts through a mechanism different from that induced by DNA damage. Unlike Adriamycin, a DNA-damaging agent that induces high levels of phosphorylation on serines 15 and 20 of p53, CP-31398 treatment of wild-type p53-expressing human tumor cells does not lead to phosphorylation of these sites on p53. Because MDM2 has been shown to play a role in the conformational switching of p53 away from the 1620-reactive form before the degradation process, the ability of CP-31398 to preserve the 1620-reactive form of p53 may render the protein resistant to MDM2-mediated degradation. This hypothesis is consistent with a mechanism by which CP-31398 not only stabilizes the active conformation but also enhances the total p53 protein levels in cells by blocking the MDM2-mediated degradation of p53.

CP-31398 was originally highlighted as a representative of a broad class of compounds that included quinazoline, acridine, and phenothiazine core structures. In an attempt to identify more potent derivatives, acridine analogues were synthesized and tested for their ability to block MDM2-mediated degradation of wild-type p53. Four acridine derivatives were found to induce p53 transcription at levels similar to or higher than CP-31398 (41). These four compounds all have acridine as the R1 group and a three-carbon linker, and different R2 groups. The anticancer mechanism of acridine derivatives remains unclear, but it is known that the mechanism is dependent on wild-type p53 and Bax. Because Bax(-/-) cells are resistant to acridine derivatives and CP-31398, the mitochondrial pathway must have a role in the mechanism of cell death induced by these compounds. It has also been suggested that acridine derivatives act by intercalating in DNA and subsequently inhibiting topoisomerase 1 or II, but like CP-31398, these compounds do not act as classic DNA-damaging agents as evidenced by lack of ser15 phosphorylation on p53 (42).

A screen of the NIH chemical library has revealed additional compounds that promote wild-type p53 activity in cells deficient for p53 activity (22). Although some of these compounds seem to function in a manner similar to CP-31398, additional compounds were identified that induce p53 target genes even in cells that do not express either mutant or wild-type p53. The activity of some of these compounds seems to hinge on their ability to induce the p73 protein (22). Although biochemical evidence has suggested that CP-31398 does not affect the p73 protein (39), compounds that induce the activity of p73 as well as p53 may have the advantage of utility in an even broader range of cancer cells.

p53 reactivation and induction of massive apoptosis. p53-stabilizing small molecules were identified based on their ability to suppress the growth of human tumor cells in a mutant p53-dependent manner (43). Screening led to the identification of p53 reactivation and induction of massive apoptosis (PRIMA-1), a low-molecular-weight compound that suppresses tumor growth by induction of apoptosis. PRIMA-1 also preserves the DNA-binding activity of wild-type p53 in cells, while restoring DNA-binding activity to His-175 and Trp-282 mutants. However, PRIMA-1 was unable to increase the Kd or maximum number of binding sites of p53-DNA binding interaction in vitro as was shown for CP-31398 and CDB3 (39). The antitumor activity of PRIMA-1 has been shown using human solid tumor xenografts (44), as well as on myeloid leukemia, where the compound is even more effective in combination with fludarabine, a commonly used antileukemia drug (45). PRIMA-1 shows a statistically significant preference for growth inhibition in human tumor cell lines containing mutant p53, rather than those containing wild-type p53. Bykov and colleagues (46) also found that PRIMA-1(MET), a methylated form that was found to be more active than the original PRIMA-1, works synergistically with other chemotherapeutic drugs to inhibit tumor cell growth. PRIMA-1(MET) combined with cisplatin synergistically induced apoptosis of tumor cells and inhibited growth of human tumor xenografts in vivo. These results indicate that combining PRIMA-1 with other anticancer agents may be a more effective form of cancer treatment.

The mechanism of PRIMA-1 action is not yet completely clear, but studies indicate that it may involve the c-Jun-NH2-kinase pathway (47). A c-Jun-NH2-kinase inhibitor blocked PRIMA-1 activity, as well as transfection with dominant-negative phosphorylation mutant c-Jun-NH2-kinase. In the same study, PRIMA-1 was found to be toxic in both premalignant and malignant cancer cell lines containing mutant p53 but not in premalignant or malignant cancer cell lines containing wild-type p53. Hsp90 α, which is induced in PRIMA-1–treated
cells, may also play an important role in the PRIMA-1 mechanism (48). Proteomic results indicate that PRIMA-1 may rescue p53-dependent transcriptional activity by restoring the p53-Hsp90 α interaction and enhancing translocation of this complex. Thus, it is possible that PRIMA-1 in combination with DNA-targeting agents may be an effective form of cancer therapy. In a similar proteomic analysis, PRIMA-1 was found to up-regulate seven proteins in breast cancer cells, all of which are involved in anaerobic glycolysis and mitochondrial intrinsic apoptosis (49). The same cells released mitochondrial cytochrome c and activated caspase-3 when treated with PRIMA-1, indicating that activation of the mitochondrial intrinsic pathway is involved in the PRIMA-1 mechanism. PRIMA-1 has been found to affect both mutant p53–containing cells and p53-null cells, indicating that there may be multiple pathways of PRIMA-1 unrelated to restoration of p53 activity that may interact with other signaling pathways (50). An important note is that PRIMA-1 activates p53 to induce apoptosis that seems to be transcription independent but Bax dependent (51). Thus, this small molecule modulator of p53 may induce apoptosis without activating transcription.

**Ellipticine.** Ellipticine is a potent anticancer agent isolated from the Australian evergreen tree of the Apocynaceae family approximately five decades ago (52). Ellipticine has been found to inhibit tumor cell growth by intercalation in DNA, inhibition of DNA topoisomerase II activity, covalent alkylation of macromolecules, and generation of cytotoxic free radicals, but the exact mechanism of action has not yet been clearly elucidated. Ellipticine and several of its derivatives have been shown to increase the levels of transcription in a mutant p53–dependent manner (53–55). Ellipticine increased transcription levels by 5- to 6-fold in the hotspot mutants 175H, 248W, 249S, 273H, and 281G. Both mutant p53–transfected cells and tumor cells with endogenous mutant p53 showed high levels of MDM2 and p21 expression when treated with ellipticine. Using the Pab1620 and Pab240 epitopes to monitor conformation, ellipticine was found to induce a shift from a mutant p53 conformation to the wild-type conformation in cells. Ellipticine affects the conformation of p53 in cells through an indirect mechanism because, unlike CP31398, ellipticine does not prevent thermodenaturation of the isolated p53 in vitro. Chromatin immunoprecipitation assays indicate that ellipticine increases DNA-binding by 4-fold in cells that express mutant p53. Ellipticine also activates endogenous mutant p53 in tumors in vivo, based on levels of MDM2 and p21 in tumor xenografts. It was also shown that many ellipticine derivatives retain the ability to activate mutant p53.

**P53R3.** P53R3, a quinazoline that is structurally unrelated to CP-31398 and PRIMA-1, was recently identified as a new mutant p53 rescuer that can induce mutant p53–dependent growth arrest in glioma cells. p53 targets, such as p21, PLIMA, and especially DR5, are induced by P53R3; therefore, tumor cells are sensitized to TRAIL-induced apoptosis (56).

**CDB3 and C-Terminal Peptides**

Several synthetic peptides have been shown to activate sequence-specific DNA binding by p53. One class of such peptides was derived from the 53BP2, a p53-binding protein involved in p53-mediated transactivation and apoptosis pathways (12). The structure of the p53 core complexed with 53BP2 is known at high resolution, and peptides were designed based on the sequence of 53BP2 that bind the p53 DNA-binding region. A second source for peptide design included regions within p53 itself that bind the core domain, including residues 363 to 393 of the COOH terminus and residues 54 to 94 of the proline-rich domain.

The peptide CDB3, derived from residues 490 to 498 of 53BP2, was found to stabilize the p53 mutant R249S by increasing its apparent melting temperature (Tm). Nuclear magnetic resonance spectroscopy and fluorescence anisotropy methods were used to define the physical site of interaction between CDB3 and the p53 DNA-binding domain (12). Interestingly, CDB3 seems to bind to the DNA-binding interface of p53 and can sterically block the function of p53. However, based on data demonstrating increased DNA binding, the authors have suggested that CDB3 acts to stabilize the DNA binding conformation of p53 but can dissociate from p53 as needed to allow for DNA binding. Treatment of p53-119ST, a highly destabilized p53 mutant, with CDB3 resulted in restoration of sequence-specific DNA-binding activity to levels near those of wild-type p53.

In a study that defined the site of p53 intramolecular interaction, a previously unidentified regulatory region of p53 was discovered: residues 80 to 93 (57). This region is necessary but not sufficient for peptide binding: it acts synergistically with residues 364 to 393 to bind the peptides. Importantly, the results indicate that COOH-terminal peptides actually bind adjacent to the p53 DNA binding domain.

Another type of p53-interacting peptide was based on a similar approach targeting the NH2 terminus of p53. Peptides were designed based on p53 amino-terminal sequences, specifically the MDM2 binding domain (58). The peptides were attached to a positively charged α-helical leader sequence to cross the cell membrane, and were introduced into human cancer cells to determine their efficacy and specificity at inhibiting cell proliferation. All three peptides were cytotoxic to human cancer cells by causing cell death, not apoptosis, and none of the peptides had an effect on the growth of normal cells. An important point is that, unlike COOH-terminal–derived peptides, cancer cells treated with NH2-terminal–derived peptides did not show increased levels in Bax or p21(WAF1/CIP1). Also, the peptides were equally effective in inhibiting growth of cells containing wild-type p53, mutant p53, or no p53. Thus, the cell death induced by the NH2-terminal–derived peptides may not be dependent on p53-induced apoptosis. For this reason, these peptides may have potential as general anticancer agents.

One barrier that limits the broad use of peptides as antitumor agents is the difficulty of crossing cell membranes to reach their target protein(s). Protein transduction domain–mediated cell entry provides a means for effective delivery of biologically active peptides into cells (59). Protein transduction domains are small cationic peptides that are capable of crossing the plasma membrane and entering the cytoplasm via a macropinocytic mechanism (60). The peptide used by Dowdy and colleagues was derived from the p53 COOH terminus (p53C) and was previously shown to induce apoptosis in cancer cells by activating wild-type p53 and restoring sequence-specific DNA-binding to p53 DNA-contact mutants.
Multidrug resistance and may provide a complementary and was shown to activate both wild-type p53 and p53 contact macromolecules that activate p53 in cells has some advantages over the small molecule approach. First, RI-TATp53C was shown to activate both wild-type p53 and p53 contact mutants, whereas small molecules may only target either wild-type or mutant p53. Because TAT-linked peptides are taken up by macropinocytosis, they are not prone to multidrug resistance and may provide a complementary and important approach in drug development.

**Functional Targeting of p53 Responses and Antitumor Effects in p53-Deficient Cells**

A functional high-throughput screening and drug development approach using noninvasive bioluminescent imaging for p53 activation or restoration of p53 function in tumor cells has been recently reported to be feasible and practical (22). Tumor cells with mutant p53 have been engineered to express a p53 firefly luciferase reporter. Any change in p53 transcriptional activity, which may come from restoration of wild-type p53 activity to mutant p53, or from activation of p53 family members, in the cells after treatment of small molecules can be captured. Using this sensitive screening method, a subset of small molecules has been identified that seemed to be able to reconstitute p53 transcriptional activity in p53-deficient tumor cells including p53-null cells, suggesting possible targets other than mutant p53 itself (Fig. 2). It has been shown that some small molecules derived from this screen increased the expression levels of human p73, and this correlated with increased expression of endogenous p53 target genes such as p21(WAF1/CIP1) or TRAIL death receptor KILLER/DR5 (16). Gene silencing of p73 was shown to block the induction of “p53-like” transcriptional responses after exposure of human tumor cells to specific small molecules but not others, consistent with the idea that p73 contributed to the functional restoration of responses normally observed when wild-type p53 is stabilized after exposure to chemotherapy or radiation (22). Small molecules derived from this screening approach were shown to harbor antitumor effects in both p53-null as well as mutant p53—expressing human colon tumor xenografts, and such effects were augmented when specific small molecules were combined with the biological agent TRAIL that induces apoptosis in part through the p53-regulated KILLER/DR5 death receptor (22). These studies provided a proof-of-principle that a functional screening approach may lead to the discovery of small molecules that can target molecular restoration of p53 or p53-like activities and that such small molecules may have potent antitumor effects that may be possible to develop further as anticancer therapeutics (Fig. 2).

**Nutlin-3a Induces p53-Dependent Apoptosis in Tumor Cells Containing Wild-Type p53 through an MDM2 Antagonistic Pathway**

The MDM2 gene is overexpressed in some human malignancies, including soft tissue sarcomas and other solid tumors such as breast cancer (62, 63). It has been proposed that inhibition of MDM2 may be an effective strategy to promote p53 activity in tumor cells that retain wild-type p53 (64, 65). Based on the crystal structure of MDM2 bound to a peptide containing the transactivation domain of p53 (66), it was shown that MDM2 contains a well-defined pocket for binding to p53 that could be exploited for drug discovery. Screening for compounds that inhibit MDM2-p53 interaction led to the identification of a group of cis-imidazoline analogues named Nutlins by Roche. Active Nutlins were found to displace p53 from its complex with MDM2 with inhibitory concentrations in the 100 to 300 nmol/L range. A crystal structure of NH2-terminal MDM2 in complex with Nutlin-3 verified that Nutlins bind to the p53-binding site on MDM2. The imidazoline scaffold of Nutlins mimics the helical backbone structure of p53. Two bromophenyl groups and one ethyl ether side chain of Nutlins are located in the hydrophobic pocket of MDM2, at sites normally occupied by residues Phe19, Trp23, and Leu26 of p53 (66). Based on the current model of p53 regulation by MDM2 (67–69), inhibition of the MDM2-p53 complex should result in (a) blockage of p53 nuclear export and degradation, causing p53 accumulation, (b) activation of MDM2 expression, and (c) activation of the p53 pathway and p53-regulated genes. These events should lead to cell cycle arrest at G1 or G2 phases, and/or apoptosis only in cells expressing wild-type p53.

Treatment of wild-type p53-containing HCT116 cells with Nutlin-1 resulted in increased levels of p53, MDM2, and p21(WAF1/CIP1), which is consistent with activation of the p53 pathway (70). Results confirm that p53 accumulation is caused by a decrease in degradation rather than an increase in expression. Treatment of cells with Nutlin-1 also increased the proportion of cells in G1 and G2 phases, and nearly depleted S phase. Nutlin-3a, the active enantiomer, shows a potent antiproliferative effect only in cells with wild-type p53. Nutlin-3a was also found to induce apoptosis in cancer cells harboring wild-type p53, using the inactive nutlin-3b enantiomer as a negative control. Nutlin-3a treatment of wild-type p53-containing tumor xenografts in mice resulted in 90% inhibition of tumor growth relative to the control.

Subsequent studies indicate that, despite 50% sequence homology in the p53-binding domains of MDM2 and MDMX, Nutlin fails to activate p53 in cells overexpressing MDMX (71). In an ELISA assay designed to detect p53-MDM2 or p53-MDMX binding, Nutlin inhibited p53-MDM2 interaction with IC50 of ~800 nmol/L. However, Nutlin did not disrupt the p53-MDMX interaction at concentrations up to 30 μmol/L (71). In a supporting study involving HDM2 and HDMX, the human homologues of MDM2 and MDMX, Nutlin-3 induced apoptosis in HDM2-transformed fibroblast cells but had no effect on HDMX-transformed fibroblasts (72). When protein levels of MDMX were reduced by shRNA, cells became sensitive to Nutlin-induced apoptosis (73). Because MDM2 and MDMX both have roles in determining p53 activation, they must both be
targeted to achieve full activation of p53 in tumor cells. A therapeutic strategy that now emerges is dual inhibition of MDM2 and MDMX.

Reactivation of p53 and Induction of Tumor Cell Apoptosis. Wild-type p53 protein in tumors can lose tumor-suppressing activity through the deregulation of MDM2 (64, 74), which binds the amino-terminus of p53, inhibits transactivation, and targets p53 for proteasomal degradation (74). The Selivanova group hypothesized that inhibition of the MDM2-p53 interaction would reactivates wild-type p53 and cause cell death in tumor cells but not in normal cells (75). A screen for p53-dependent tumor suppressor activity in two isogenic cell lines differing only in p53 status led to the identification of compounds that rescue p53 activity irrespective of the mechanism (Fig. 2). Further experiments indicated that the molecular mechanism of one of these compounds, reactivation of p53 and induction of tumor cell apoptosis (RTA), involves high-affinity binding to the NH2-terminal domain of p53. RTA is a cell-permeable small molecule that was found to induce accumulation of p53 in tumor cells. RTA blocked p53-MDM2 interaction in vitro and in vivo, and blocked p53 ubiquitination by MDM2 in vitro. In tumor cells, RTA was found to restore p53 transactivation activity and induce p53-dependent apoptosis. In vivo, RTA showed strong antitumor activity, with >2-fold reduction in the growth rate of p53-positive xenografts and no effect in p53-null xenografts. Based on these data, RTA has potential for development as an anticancer drug that target tumors with wild-type p53.

Blockage of the p53-MDM2 interaction in vitro required preincubation of p53 with RTA, suggesting a two-step mechanism that has been previously described for other small molecules (76). The Selivanova group hypothesized that RTA binds the p53 NH2-terminal region with fast kinetics, and then undergoes a slow conformational change in the second step that prevents MDM2 binding (75). Overall, the data suggests that RTA induces an active conformation of p53 that has low affinity for negative regulators. However, Krajewski and colleagues (77) later used nuclear magnetic resonance to monitor the effect of RTA on the p53-MDM2 complex and reported that RTA may not block p53-MDM2 binding in vitro. Up to a 5-fold molar excess of RTA relative to p53 did not prevent the formation of the p53-MDM2 complex. In these experiments, RTA was either preincubated with p53 before MDM2 was added, or was titrated into p53-MDM2. The Selivanova group suggested that the protein purification procedure affects the binding of RTA to p53 in vitro, and that RTA binds only the wild-type conformation of p53. Also, small RTA-induced shifts in the spectra of the p53 core domain reported by Krajewski and colleagues (77) may support the Selivanova group’s data that the conformational change induced by RTA in p53 propagates to the p53 core domain.

Benzodiazepinediones, MI-63, and MI-43. Benzodiazepinedione (78, 79) and MI-63 (80) are wild-type p53 activators that interfere with MDM2-p53 binding. Benzodiazepinedione derivatives were identified from screening chemical libraries by a miniaturized thermal denaturation assay. The X-ray crystal structure revealed that benzodiazepinediones interact with the p53-binding pocket of MDM2. Optimized Benzodiazepinedione derivatives have been shown to increase the transcription of p53 target genes and decrease proliferation of tumor cells expressing wild-type p53.

MI-63 (MDM2 inhibitor 63), unlike nutlins and benzodiazepinediones that mimic only the Phe19, Trp23, and Leu26 residues in p53 and their interactions with MDM2, was designed considering a fourth residue (80). Leu22 in p53 that was also shown to play a role for the interaction between p53 and MDM2. Indeed, the new compound MI-63 binds to MDM2 more efficiently with a Ki value of 3 nmol/L. 12 times more potent than Nutlin-3, p53 transcriptional activity was induced by MI-63 in wild-type p53 expressing human solid tumor cells and leukemia cells (81). Another member in the same MI family, MI-43, shows a similar MDM2 inhibition effect and induces apoptosis in lung cancer cells (82).

RETRA. A small molecule named RETRA was identified to selectively kill tumor cells carrying mutant p53 (83). Further investigation showed that RETRA dissociates p73 from mutant p53, executes transcriptional activity, and induces expression of p53 targets. Knockdown of p73 by siRNA attenuates RETRA function in cancer cells. In vivo, an antitumor effect was observed.

Tenoovins. Another screen by a group in the United Kingdom identified a bioactive compound called tenovin-1, which induces stability of wild-type p53 protein and subsequently induces p53-dependent cell cycle arrest and apoptosis, and exhibits an in vivo antitumor effect. Tenoovin-1 and a more water-soluble analog, tenovin-6, inhibit NAD-dependent deacetylase, SirT1, which has been reported to destabilize p53 protein by catalyzing the deacetylation of p53 at lysine 382. Unlike DNA-damaging agents, which also induce acetylation of p53 at lysine 382 and other sites, tenovins do not induce DNA damage signaling leading to phosphorylation of H2AX (84).

Taxanes and metformin. Due to the high percentage of human tumors with mutated p53, agents that preferentially inhibit the growth of cells lacking p53 function have been sought in recent years. Although several such agents have been identified through the screening of the National Cancer Institute tumor cell panel, the mode of action for these agents remains poorly understood (85). Paclitaxel, which is the only widely used drug to preferentially kill cells with mutated p53, seems to act as an inhibitor of microtuble polymerization. Studies from Hait’s group have established that the transcriptional activity of p53 is directly related to tumor cell sensitivity to taxanes. This effect seems to be mediated by microtubule-associated protein 4, which occurs when p53 is mutated, increases microtubule polymerization, fluorescein-labeled paclitaxel binding, and sensitivity to paclitaxel (86).

Metformin, a drug used to enhance insulin sensitivity in diabetic patients, has also been shown to selectively induce apoptosis in cells that are devoid of p53 activity (87). The drug acts in part by increasing AMP kinase activity, enhancing fatty acid β-oxidation, and inhibiting oxidative phosphorylation. Cells with wild-type p53 are able to compensate for the suppression of oxidative phosphorylation by increasing the rate of glycolysis, whereas the pathway is maximally functioning to sustain the energy requirement of tumor cells with mutated p53. Because tumor cells with mutated p53 are unable to alter their metabolic pathway, they may be more severely affected by the effects of metformin.
Pifithrin Reversibly Blocks Wild-Type p53 Activity During Cancer Treatment to Prevent Damage to Normal Tissues

In response to cell stress signals such as chemotherapy and radiation, p53 induces cell cycle arrest or apoptosis. Thus, an unfortunate side effect of anticancer therapy is the damage of normal tissues (88), and it has been proposed that temporary suppression of p53 during treatment of p53-deficient tumors could prevent this side effect (89). A recent study by Evan and colleagues (20) used a mouse model in which the p53 status could be reversibly switched between functional and inactive states to explore the cause-and-effect relationship between the acute pathologic response to DNA damage and suppression of consequent tumors. Results show that the acute p53-dependent pathology induced by systemic genotoxic injury may be irrelevant for suppression of tumors that emerge subsequent to the time of injury. Delaying restoration of p53 activity until after the DNA-damage response has subsided results in tumor suppression activity dependent on the p19ARF pathway. These results indicate that reversible pharmacologic inhibition of p53 during, or shortly after, genotoxic injury may help to alleviate the negative side effects of cancer therapy while still retaining tumor suppression activity that is induced by unregulated oncogene action.

Pifithrin-α ("p-fifty three inhibitor"). PFT-α, is a small molecule that has been found to inhibit p53-dependent transactivation without affecting cell growth or survival rates (21). PFT-α inhibits apoptotic death in cells that undergo rapid p53-dependent apoptosis in response to various treatments, including UV light and γ radiation. The antiapoptotic activity of PFT-α is dependent on p53 because no effect has been observed in p53-deficient cells. Also, PFT-α significantly increases the percentage of G2-arrested cells after γ irradiation but has no effect on nonirradiated cells. Data from protein immunoblotting in combination with two-dimensional protein analysis and gel assays indicates that PFT-α has no effect on phosphorylation or sequence-specific DNA-binding activity of p53. It was also found that PFT-α decreases the levels of nuclear p53 induced by UV irradiation but has no effect on cytoplasmic levels of p53. Based on these data, it has been hypothesized that the mechanism of PFT-α involves nuclear import and/or export of p53, or destabilization of nuclear p53. Using a 14C-thymidine incorporation assay to analyze DNA synthesis in treated versus untreated mice, the labeling of tissues was significantly reduced after γ irradiation in p53(+/+) mice but not p53(-/-) mice. This suggests that, after whole-body irradiation, PFT-α attenuates the p53-dependent block of DNA replication in rapidly growing tissues. PFT-α has potential for reducing side effects of anticancer therapy, and because its effects are p53-dependent, the sensitivity of tumors to treatment would not be compromised. The blocked inhibition of DNA synthesis in γ-irradiated wild-type p53-expressing mice was correlated with the ability of the mice to survive a lethal dose of ionizing radiation that p53-null mice are able to survive. However, blockage of p53 activity in normal cells gives increase to concerns about genomic instability after PFT-α treatment, which may be monitored and minimized by short term exposure to PFT-α before or during radiation.

Conclusions

Therapeutic modulation of wild-type and mutant p53 proteins and downstream signaling remains a high priority for anticancer drug development. Insights into potential approaches to achieving this have come from genetic, biochemical, and functional studies of wild-type and mutant p53 proteins. A number of small molecules and peptides have been identified and showed to possess antitumor activity. As toxicity is part of the equation in optimizing the therapeutic window, insights have recently emerged into how to best minimize adverse side effects with small molecules that protect normal tissues from radiation damage. Continued focused efforts are required to make progress in the identification and development of novel therapeutics targeting the p53 signaling pathway and the p53 family.

Disclosure of Potential Conflicts of Interest

W.S. El-Derhy is the Founder and Chair of the Scientific Advisory Board of Oncogenics, Inc., a biotech company focused on developing novel small molecule anticancer therapies targeting mutant p53 protein. F. Rastinejad is the Chief Scientific Officer of Oncogenics, Inc. The other authors disclosed no potential conflicts of interest.

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