A Single Mutant, A276S of p53, Turns the Switch to Apoptosis
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ABSTRACT: The tumor suppressor protein p53 induces apoptosis, cell cycle arrest, and DNA repair along with other functions in a transcription-dependent manner [Vousden, K. H. Cell 2000, 103(5), 691−694]. The selection of these functions depends on sequence-specific recognition of p53 to a target decameric sequence of gene promoters [Kitayner, M.; et al. Mol. Cell 2006, 22(6), 741−753]. Amino acid residues in p53 that directly bind to DNA were analyzed, and the replacement of A276 in p53 with selected amino acids elucidated its importance in promoter transcription. For most apoptotic and cell cycle gene promoters, position 9 of the target decameric sequence is a cytosine, while for DNA repair gene promoters, thymine is found instead. Therefore, selective binding to the cytosine at the ninth position may transcribe apoptotic gene promoters and thus can induce apoptosis and cell cycle arrest. Molecular modeling with PyMOL indicated that substitution of a hydrophilic residue, A276S, would prefer binding to cytosine at the ninth position of the target decameric sequence, whereas substitution of a hydrophobic residue (A276F) would fail to do so. Correspondingly, A276S demonstrated higher transcription of PUMA, PERP, and p21WAF1/CIP1 gene promoters containing a cytosine at the ninth position and lower transcription of GADD45 gene promoter containing a thymine at the ninth position compared to wild-type p53. Cell cycle analysis showed that A276S maintained similar G1/G0 phase arrest as wild-type p53. Additionally, A276S induced higher apoptosis than wild-type p53 as measured by DNA segmentation and 7-AAD assay. Since the status of endogenous p53 can influence the activity of the exogenous p53, we examined the activity of A276S in HeLa cells (wild-type endogenous p53) versus T47D cells (mutated and mislocalized endogenous p53). The same apoptotic trend in both cell lines suggested A276S can induce cell death regardless of endogenous p53 status. Cell proliferation assay depicted that A276S efficiently reduced the viability of T47D cells more than wild-type p53 over time. We conclude that the predicted preferred binding of A276S to cytosine at the ninth position better transactivates a number of apoptotic gene promoters. Higher induction apoptosis than wild-type p53 makes A276S an attractive candidate for therapy to eradicate cancer.

KEYWORDS: A276S, p53, apoptosis, cell cycle arrest, T47D, tumor suppressor

INTRODUCTION
The tumor suppressor protein p53, also known as the death star, is the natural nemesis of cancerous cells.1,3 The DNA-binding domain (DBD) binds selectively to double-stranded DNA to execute the destined function of p53. From a cancer therapeutic point of view, cell cycle arrest and apoptosis are the two most desirable functions of p53 to stagnate and to eradicate cancer cells, respectively.1 However, it remains unclear how the specific DBD–DNA interaction determines p53’s route of action (Figure 1): apoptosis, cell cycle arrest, or DNA repair?

The target DNA-binding site for p53 consists of two half-sites with a decameric base pair motif of 5′-RRRCWWGYYY-3′ (R = purine; W = A/T; Y = pyrimidine) separated by a 0−13 base pair spacing.6 Several crystallographic structures of the p53-DBD complex with various target DNA half-site palindromes were studied to identify the key residues involved in DNA recognition.7,8 Seven residues, K120, S241, R248, R273, A276, C277, and R280 are reported present at the p53 DBD–DNA interface that make direct contacts with the DNA half-sites.7 Therefore, we investigated if any of these direct DNA-binding residues can be altered to improve either apoptosis or cell cycle arrest. S241, R248, and R273 were found to make nonspecific contacts with the phosphate backbone of DNA for docking; likewise, R280 anchors nonspecifically to the major groove of DNA.2 This leaves A276, C277, and K120 as critical direct DNA-binding residues that select specific DNA sequences and determine the functional pathway of p53. Of these three, K120 acetylation was found to be crucial for induction of apoptosis but not involved in cell cycle arrest. Correspondingly, the mutation of this residue (the so-called hot-spot mutant K120R) in certain cancers allows these tumors to evade apoptosis.9 Therefore, sans post-translational modification, A276 and C277 are the likely candidates that determine the outcome of the p53 DBD–DNA interaction. Other than speculation, no experiments have directly addressed the degree of involvement of...
A276 or C277 in apoptosis or cell cycle arrest. Since the thiol (SH) group of cysteine is known to form disulfide bonds\textsuperscript{10} and cysteine itself participates in numerous post-translational modifications,\textsuperscript{11} we decided to refrain from mutating C277 due to its critical functions and narrowed our focus to A276 of p53.

Systematic analysis of the DNA-binding sites of p53 with all known promoters showed that p53 has a higher binding affinity for gene promoters involved in cell cycle, DNA repair than apoptotic promoters.\textsuperscript{12} On the basis of this observation, the crystal structure of p53 DBD bound to two sets of palindromic DNA half-sites with either cytosine (C9) or thymine (T9) located at the ninth position was solved to understand these specific interactions.\textsuperscript{2} Sequential inspection of these promoter regions revealed that most apoptotic gene promoters and the 3′-promoter site of the cell cycle gene, p21, have cytosine (C) in their ninth position of the target decameric sequence, whereas the rest of the promoters such as DNA repair and other cell cycle genes have thymine (T). In wild-type p53, the methyl side chain (carbon 8) of A276 contacts the methyl group (carbon 7) of T9 via a hydrophobic interaction as depicted in Figure 2A. However, in the presence of C9, A276 moves away from the hydrophilic site and is not involved in interacting with DNA (Figure 2B).

If alanine is substituted with a hydrophilic residue such as serine, this may now interact with C9, whereas substitution with a more hydrophilic residue such as phenylalanine may interact better with T9. Modeling of A276 bound to the DNA half-site was visualized as a stick model and color-coded to distinguish it from the rest of the structure. The DNA half-site contains either an A/T or G/C base pair at the ninth position; PyMOL Mutagenesis Wizard was used to generate A276S and A276F mutations associated with this DNA half-site. Both backbone-dependent and backbone-independent rotamers were simulated at 0.3 fps to obtain the least-hindered conformation of the mutated residues. In addition to stericity, the conformation with the closest possible distance from the target nucleotide unit (A/T or G/C) was selected to generate the representative images in Figure 2.

Construction of Plasmids and Mutagenesis. EFGP-p53 (pEGFP-p53) plasmid was constructed as previously described.\textsuperscript{13} E-p53\textsubscript{ΔTD} (amino acids 1–322) plasmid was truncated at BspEII and KpnI restriction sites. Using site-directed mutagenesis, EFGP-p53 plasmid was mutated to create A276-variant p53 with the following mutagenic primers: A276S, forward 5′-CTTTGAGGTGCGTGTTTGTCCCTGTCCTGGGAGAGACCGGCG-3′ and A276S; backward 5′-CGCCGGTCTCTCCCAGGACAGGAACAAACACGCACCTCAAG-3′, A276G, forward 5′-TTTGAGGTGCGTGTTTGTGGCTGTCCTGGGAGAGACCGGC-3′, and A276G; backward 5′-GCCGGTCTCTCCAGGACAGAAGAACAAACACGCACCTCAAG-3′, A276F, forward 5′-CTTTGAGGTGCGTGTTTGTCTGTCCTGGGAGAGACCGGCG-3′, and A276F; backward 5′-GCCGGTCTCTCCCAGGACAGGAACAAACACGCACCTCAAG-3′, A276R, forward 5′-CTTTGAGGTGCGTGGTTTGTCTGTCCTGGGAGAGACCGGCG-3′, and A276R; backward 5′-GCCGGTCTCTCCCAGGACAGGAACAAACACGCACCTCAAG-3′.
Figure 2. Stick model of p53-A276 interacting with DNA. Light gray color indicates wild-type (WT) p53 residues surrounding A276 position; green indicates carbon; blue indicates nitrogen; red indicates oxygen; white indicates hydrogen; and orange represents phosphorus. Residues circled in black indicate hydrophobic sites, and squares indicate hydrophilic sites. C9 represents cytosine located at the ninth position of p53, and T9 is thymine at the ninth position. (A) A276:T9. The hydrophobic interaction between carbon 8 of A276 (circled) and carbon 7 of T9 (circled) is depicted with the orange dashed line. (B) A276:C9. Replacement of T9 with C9 nucleotide shows loss of interaction between A276 and target DNA. (C) A276S:T9; substitution of alanine with serine. The OH group of serine moves away from the hydrophobic carbon 7 of T9 (circled). (D) A276S:C9. The square shows possible hydrogen bonding or water-mediated interaction between the OH-group of A276S and nitrogen 4 of C9 as illustrated with purple dashed lines. In addition, A276S may form hydrogen bonds with both phosphate groups of G8 and C9. (E) A276F:T9; substitution of alanine with phenylalanine. The benzene ring of phenylalanine serves as a hydrophobic pocket for the circled methyl group of T9. (F) A276F:C9. The aromatic ring of A276F is repulsed from the hydrophilic environment of G8 and C9.
Cell Lines and Transient Transfections. Human cervical carcinoma HeLa cells (ATCC, Manassas, VA) were grown as monolayers in RPMI (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-glutamate (Inovitrogen), and 0.1% gentamicin (Inovitrogen). This was used as the growth medium for human ductal breast epithelial cells T47D (ATCC) and was further supplemented with 4 mg/L of insulin (Sigma, St. Louis, MO). All cells were maintained in a 5% CO2 incubator at 37 °C. Three × 10^4 cells for HeLa cells were plated in 6-well plates for most assays (Greiner Bio-One, Monroe, NC) or in 2-well live cell chambers for fluorescence microscopy (Nalge Nunc, Rochester, NY), unless stated otherwise. Twenty-four hours after seeding, the cells were transfected with 3.5 μg of DNA per well using Lipofectamine 2000 (Inovitrogen) per manufacturer’s instructions as previously described.13

Luciferase Assay. All constructs (3.5 μg of DNA) were transfected in T47D cells with either p53-Luc cis-reporter (Agilent), pBV-Luc PUMA reporter (a generous gift from Dr. Vogelstein, Addgene plasmid 16591),14 pGL3-Luc GADD45WT reporter (a generous gift from Michael Greenberg, Addgene plasmid 8356),15 pGL3- Basic PEP reporter (a generous gift from Dr. T. Jacks, Addgene 14810),16 or pBluescript II KS (+) + luc p21WAF1/CIP reporter (a generous gift from Dr. Phillip Moos, University of Utah) was cotransfected with plasmid encoding renilla luciferase (a generous gift from Dr. P. Moos, University of Utah) with 1.5 μg of each construct in duplicate. Western blot analysis, 24 h following transfection of T47D cells were tested after 48 h, and T47D cells were transfected after 48 and 72 h, respectively, following transfection. The samples were analyzed using the FACScanto-II (BD-BioSciences, Palo Alto, CA) and antiactin (ab3280, Abcam) were detected with antimouse HRP-conjugated secondary antibody (ab6814, Abcam) before the addition of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). Bands were detected with a FluorChem FC2 imager (Alpha Innotech, Santa Clara, CA).

7-AAD Assay. Hela and T47D cells were stained with 7-aminoactinomycin D (7-AAD, Invitrogen) according to the manufacturer’s instructions. HeLa cells were tested after 24 h, and T47D cells were tested after 48 and 72 h, respectively, following transfection. The samples were excited with a 488 nm laser. Sample analysis was based on EGFP positive cells, and all samples were gated at same EGFP intensity levels. All constructs were tested in three independent experiments (n = 3).

Cell Cycle Arrest. For cell cycle arrest analysis, 1 × 10^6 T47D cells were transfected as described above (Cell Lines and Transient Transfection). Forty-eight hours post-transfection, adherent cells were collected, washed in PBS (Invitrogen), and fixed in 70% cold ethanol at −20 °C overnight. Fixed cells were centrifuged at 2000 rpm for 5 min and washed three times with PBS. Cells were then suspended in stain solution containing 10% v/v of 0.5 mg/mL propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO), 5% v/v PBS, 84% v/v of 4 mM citrate buffer (Sigma-Aldrich), 0.62% v/v RNase A (Sigma-Aldrich), 1% v/v Triton X-100 (Sigma-Aldrich), and 3% w/v of PEG 6000 (Fluka, St. Louis, MO) for 20 min at 37 °C in the dark. An equal volume of salt solution containing 5% of PI, 1% Triton X-100, 89% v/v 0.4 M NaCl (Thermo Fisher Scientific Inc., Pittsburgh, PA), and 3% m/v of PEG 6000 was added to the stained cells and incubated at 4 °C for 1 h in the dark as well. Cells were gated at the same intensity level of EGFP. EGFP and PI were excited at 488 nm and detected at 507 and 670 nm, respectively. Samples were repeated in three distinct experiments (n = 3).

Statistical Analysis. Data were represented as mean ± standard deviation and analyzed using one-way ANOVA with Tukey’s posthoc test. Statistical differences between the same construct at different time points was compared via two-way ANOVA with Bonferroni test using GraphPad Prism software. A p value < 0.05 was considered significant.
RESULTS

Selection of Amino Acids for A276 Mutation. In order to experimentally verify the importance of A276, wild-type p53 was mutated at this position to the select amino acids with side chains varying in nature, size, charge, and hydrophobicity (Table 1). Even though the side chains of both alanine (A) and phenylalanine (F) are hydrophobic, they differ in size and degree of hydrophobicity. The smallest amino acid, glycine (G), lacks a side chain and therefore can elucidate the necessity of a side chain at the 276th position. Compared to only three possible hydrogen bonds with serine (S), the amide group of asparagine (N) can form five potential hydrogen bonds simultaneously. Despite both being neutral, serine and asparagine differ in size and hydrophilicity. Glutamic acid (E) and arginine (R) are negatively and positively charged residues, respectively, that possess the ability to make, if any, salt bridges with neighboring charged residues. Overall, this selected set of mutations approximates all the side chain properties of standard amino acids.

A276S Shows Elevated Transcriptional Activity of a Promoter Containing C9 at the DNA Half-Site. To screen and compare the transcriptional activity, we subjected all p53 mutants (listed in Table 1) to the p53 cis-reporter system. Upon p53 recognition to its repeated synthetic promoter consensus (TGGCCTGACCTTGCCCTGG)14,25 p53 firefly luciferase is expressed in proportion to the binding of p53 to the reporter. As depicted in Figure 3, all constructs failed to display transcriptional activity compared to the negative control (EGFP) with the exception of A276S and wild-type p53. As previously mentioned, the negative control (EGFP) and A276F showed minimal transcriptional activity (Figure 3). Enhanced transcription of these gene promoters suggests that A276S can better induce the mediators of both p53-dependent intrinsic and extrinsic apoptotic pathways than wild-type p53.

A276S Transactivates Apoptotic and Cell Cycle Genes. To investigate whether the preferred C9 binding of A276S translates into elevated transactivation, we compared transcriptional activity from several different p53 target gene promoters in T47D cells. These selected genes, PUMA, PERP, p21WAF1/CIP1, and GADD45 have defined decameric promoter sequences and are known to be involved in diverse p53-mediated functions as listed in Table 2. Like the p53-binding reporter above, the promoters of these genes are fused to luciferase. The resultant lucinescence can therefore indicate the transcriptional activity of target gene promoters upon binding to wild-type or mutant p53.

PUMA and PERP are apoptotic genes targeted by p53. Once expressed, PUMA translocates to the mitochondria and interacts with antiapoptotic Bcl-XL and Bcl-2 proteins to mediate rapid apoptosis via the intrinsic pathway.14 PERP (PMP-2) is a membrane-bound protein that acts as a cell death receptor and is speculated to be important for the extrinsic apoptotic pathway.16 A276S displayed significantly higher PUMA (Figure 4A) and PERP (Figure 4B) gene promoter transcription than wild-type p53. As previously mentioned, the negative control (EGFP) and A276F showed minimal transcriptional activity (Figure 3). Enhanced transcription of these gene promoters suggests that A276S can better induce the mediators of both p53-dependent intrinsic and extrinsic apoptotic pathways than wild-type p53.

A276 denotes alanine at the 276th position of p53 followed by the one letter code of the mutated amino acid residue. Positive values represent hydrophobic side chains, negative values represent hydrophilic side chains, and 0 represents no side-chain. The charges on the side chains are positive, neutral, or negative.

Table 1. Properties of Mutated Amino Acids

<table>
<thead>
<tr>
<th>mutation</th>
<th>amino acid characteristics</th>
<th>side-chain charge (pH 7.4)</th>
<th>size (kDa)36</th>
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<tr>
<td>A276F</td>
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<td>1.108.05</td>
<td>-5</td>
</tr>
<tr>
<td>A276N</td>
<td>hydrophilic</td>
<td>neutral</td>
<td>1.132.12</td>
<td>-25</td>
</tr>
<tr>
<td>A276E</td>
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<td>negative</td>
<td>1.147.13</td>
<td>-60</td>
</tr>
<tr>
<td>A276R</td>
<td>hydrophobic</td>
<td>positive</td>
<td>1.174.10</td>
<td>-75</td>
</tr>
</tbody>
</table>

“A276 denotes alanine at the 276th position of p53 followed by the one letter code of the mutated amino acid residue. Positive values represent hydrophobic side chains, negative values represent hydrophilic side chains, and 0 represents no side-chain. The charges on the side chains are positive, neutral, or negative.

Figure 3. Luciferase assay: Relative luminescence illustrates the ability of mutated p53 constructs to activate the p53 cis-reporter in T47D cells. Mean values were analyzed using one-way ANOVA with Tukey’s post test (***p < 0.001). Error bars represent standard deviations from independent experiments (n = 3).
Figure 1 to Figure 4C), validating the reliability of this reporter assay for the expression of other genes.

GADD45 stimulates DNA repair excision upon p53 transcription. However, the promoter of GADD45 gene

Table 2. p53-Consensus for Target Decameric Sequences

<table>
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<th>promoter consensus</th>
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<td>p53 recognition</td>
<td>binding consensus</td>
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<td>PUMA</td>
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<td>G</td>
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<tr>
<td>p21</td>
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<td>G</td>
</tr>
<tr>
<td>GADD45</td>
<td>DNA repair</td>
<td>A</td>
</tr>
</tbody>
</table>

The top row represents decameric sequence (R = A or G; W = A or T; Y = C or T).12 The 9th positions of promoters are either C or T. Lowercase letters in this table represent exceptions to the consensus. References: PUMA;14 p21WAF1/CIP1;26 PERP;27 GADD45.28

Figure 4. Transcription of (A) PUMA, (B) PERP, (C) p21WAF1/CIP1, and (D) GADD45 promoters in luciferase system. Mean values are analyzed with one-way ANOVA with Tukey’s post test (*p < 0.05; **p < 0.01; ***p < 0.001). Error bars represent standard deviations from independent experiments.

Figure 5. Cell cycle arrest: all constructs were assessed for DNA content 48 h post-transfection in T47D cells. E-p53ΔTD represents monomeric p53 as a negative control instead of EGFP. The values reported are averaged DNA content ± SD of transfected cells in G0/G1 phase. (A) p53ΔTD, (B) E-p53 (wild-type), (C) A276S, and (D) A276F. Statistical analysis was performed using one-way ANOVA with Tukey’s post test.
contains a thymine (Table 2, bottom row) at position 9 instead of cytosine. As expected, A276S showed lower transcription of GADD45 gene promoter compared to wild type p53 (Figure 4D).

**A276S Maintains Cell Cycle Arrest.** To further evaluate the CDK inhibitory effect of increased p21<sub>WAF1/CIP1</sub> expression, we examined the cell cycle arrest potential of each construct. E-p53TD lacks the tetramerization domain (TD); thus, it resides as an inactive monomeric form in the nucleus. Since our cell cycle analysis method detects the nuclei, we used monomeric E-p53TD as a negative control to be able to gate for EGFP transfected cells. Wild-type p53 significantly arrests cells in G0/G1 phase better than p53ΔTD (p < 0.01) (Figure 5, compare A to B) as expected. Although the p21<sub>WAF1/CIP1</sub> expression profile of A276S was higher than wild-type p53, it demonstrated similar cell cycle arrest (Figure 5C). It can be speculated that, after a certain threshold level, any further expression of p21<sub>WAF1/CIP1</sub> may not contribute to increased cell cycle arrest. A276F also succeeded to arrest cells better than the negative control (Figure 5D). Other than p21<sub>WAF1/CIP1</sub>, A276F may bind to several p53 target genes such as B99, cyclin-G, BDS, and GML<sup>12</sup> or via an unknown pathway to induce the observed cell cycle arrest.

**A276S Inhibits Cell Proliferation.** Next, the improved transcriptional activity of A276S was analyzed for its effect in T47D cells. As increased expression of apoptotic genes and cell cycle arrest at G0/G1 phase are expected to stagnate growth, cell proliferation was monitored by cell counts after 48 and 72 h following transfection using trypan blue exclusion. As seen in Figure 6, 48 h post-transfection, A276S (2nd bar) and wild-type p53 (1st bar) similarly reduced cell proliferation compared to wild-type p53. A276F (4th bar) and EGFP (1st bar) failed to achieve significant cell death. Moreover, each construct was compared at different time points. A276S induces higher cell death at 72 h (7th bar) than at 48 h (3rd bar) following transfection. Wild-type p53 remained statistically indistinguishable at both time points. Since T47D cells have mutated and mislocalized endogenous p53, constructs were also tested in HeLa cells with endogenous wild-type p53. In HeLa cells, the 7-AAD assay was conducted at 24 h due to their higher sensitivity to apoptosis. As observed in Figure 8B, A276S successfully displayed the same apoptotic trend, regardless of endogenous p53 status. EGFP and A276F (1st and 4th bar, respectively, Figure 8B) behaved as expected and failed to induce apoptosis. This result, together with apoptotic promoter transcription and nuclear segmentation, establishes that A276S induces higher apoptosis than wild-type p53.

Figure 6. Viable T47D cells after 48 and 72 h following transfection. The number of proliferating cells for each construct was normalized to the number of EGFP transfected viable cells (negative control). Values are reported as mean ± SD; one-way ANOVA with Tukey’s post test (*p < 0.05; **p < 0.01; ***p < 0.001).

p53 (1st bar) similarly reduced cell proliferation compared to A276F (3rd bar). However, at 72 h, A276S (5th bar) caused a significant reduction in cell proliferation compared to wild-type p53 (4th bar). Not surprisingly, A276F showed significant reduction in cell viability after 72 h in agreement with the cell cycle arrest observation. This provides the evidence (albeit indirect) that over time A276S effectively employs cell death and cell cycle arrest together to inhibit cell proliferation.

**A276S Educes Hallmark of Apoptosis.** Analysis of cell morphology is a widely used method to visualize the induction of apoptosis.<sup>13</sup> Among these hallmarks, nuclear segmentation can be quantified to measure apoptosis of transfected cells. Cells morphologies of different constructs were photographed as depicted in Figure 7A. The averaged segmented nuclei were quantified and graphed as shown in Figure 7B. Congruent to the previous experiments, A276S demonstrated a higher percentage of segmented cells versus wild-type p53. A276F behaved similar to EGFP (Figure 7B). With elevated expression of PUMA, PERP, and p21<sub>WAF1/CIP1</sub>, indeed A276S induces apoptosis better than wild-type p53.

**A276S Executes Higher Cell Death than Wild-Type p53.** To confirm the end-point of apoptosis, we conducted a flow cytometric experiment with 7-AAD, which measures late apoptosis or necrosis. The percentage of 7-AAD positive T47D cells was measured 48 and 72 h post-transfection as depicted in Figure 8A. At both time points, A276S (3rd bar and 7th bar) demonstrated significantly higher late apoptosis than wild-type p53 (2nd bar and 6th bar). A276F (4th bar) and EGFP (1st bar) failed to achieve significant cell death. Moreover, each construct was compared at different time points. A276S induces higher cell death at 72 h (7th bar) than at 48 h (3rd bar) following transfection. Wild-type p53 remained statistically indistinguishable at both time points. Since T47D cells have mutated and mislocalized endogenous p53, constructs were also tested in HeLa cells with endogenous wild-type p53. In HeLa cells, the 7-AAD assay was conducted at 24 h due to their higher sensitivity to apoptosis. As observed in Figure 8B, A276S successfully displayed the same apoptotic trend, regardless of endogenous p53 status. EGFP and A276F (1st and 4th bar, respectively, Figure 8B) behaved as expected and failed to induce apoptosis. This result, together with apoptotic promoter transcription and nuclear segmentation, establishes that A276S induces higher apoptosis than wild-type p53.

**DISCUSSION**

Analysis of the crystallographic structure of p53 bound to DNA revealed several direct DNA-binding residues. Among these, L120, A276, and C277 are reported as the three critical residues for selecting target promoters. A previous study showed that A276 in p53 specifically interacts with the ninth position of the target DNA decameric sequence.<sup>7</sup> The methyl group of A276 was found to interact with the methyl of group of thymine via a hydrophobic interaction. However, in the presence of a cytosine at position 9 of a target decameric sequence, the methyl group of A276 did not interact with the hydrophilic N7 site. Interestingly, another key study reported that a number of apoptosis and cell cycle arrest gene promoters have a cytosine at position 9, while for most DNA repair gene promoters, thymine is found instead.<sup>12</sup> Since our goal is to improve p53 as a cancer therapeutic, we were interested in modifying p53 to improve its apoptotic function. From this, we hypothesized that substitution of a hydrophilic residue (A276S) would selectively increase binding to C9 of apoptotic gene promoters. Therefore, the A276S mutant should induce higher apoptosis than wild-type p53, yet maintain cell cycle arrest.

Molecular modeling was performed to visualize the possible interactions between wild-type p53, A276S, or A276F and the p53-target decameric sequence containing either a C9 or a T9. As opposed to wild-type p53, A276S favored binding to the decameric sequence at position 9 containing a cytosine instead of a thymine. We speculated that the hydroxyl (OH) group of serine might be advantageous over the methyl group of alanine to form a hydrogen bond with the nitrogen of C9. In addition, the lone pair of electron from oxygen might make water-mediated hydrogen bonds with the phosphate backbone of the target DNA. With significant steric hindrance, mainly because
of size, A276F likely prefers binding to the decameric sequence with a thymine at position 9 rather than a cytosine.

A276S was selected as a promising candidate to bind better to promoters containing C9. Failure of other selected amino acids (F, G, S, N, E, and R) signified the importance of the 276th position for promoter transcription, identifying A276 as a hot spot for p53. A276F was chosen as a negative control, in addition to EGFP, due to modeling results and initial lack of activity in a p53 reporter gene assay.

The benefit of enhanced binding of A276S to C9 was tested in T47D cells by measuring the transcription of selected gene promoters using a luciferase-based assay. Four gene promoters were tested: PUMA, PERP, p21<sup>WAF1/CIP1</sup>, and GADD45. PUMA expression leads to the intrinsic apoptotic pathway, PERP follows the extrinsic apoptotic pathway, p21<sup>WAF1/CIP1</sup> modulates cell cycle arrest as well as apoptosis, and GADD45 repairs DNA damage. A276S demonstrated significantly higher transcription of PUMA, PERP, and p21<sup>WAF1/CIP1</sup> gene promoters and lower transcription of GADD45 promoter compared to wild-type p53. As expected, the negative controls, A276F and EGFP, failed to transactivate these gene promoters. Up-regulation of these genes suggested the possible mechanisms by which A276S could induce higher apoptosis than wild-type p53 and maintain cell cycle arrest.

The outcome of increased p21<sup>WAF1/CIP1</sup> expression (a CDK inhibitor) was examined in cell cycle regulation. All three constructs, wild-type p53, A276S, and surprisingly A276F, displayed similar cell cycle arrest. As anticipated, the negative control (monomeric p53ΔTD) apprehended less G0/G1 DNA content than all other constructs. It is thought that over-expression of p21<sup>WAF1/CIP1</sup> after a threshold point may mediate cell death rather than cell cycle arrest. In addition, A276F might bind to other cell cycle gene promoters or undergo an unknown pathway that leads to the observed cell cycle arrest.

A276S also inhibited cell proliferation over an extended time period. Cell counts normalized to EGFP showed that A276S maintained the same number of viable cells at 42 h and 72 h, respectively. Even though initially the same as A276S, wild-type p53-treated cells were more viable over time. This indirectly demonstrated that A276S can efficiently implement apoptosis and cell cycle arrest versus wild-type p53. In agreement to the observed cell cycle experiment, A276F also reduced cell proliferation over time to some extent (although not as potently as A276S or wild-type p53).

Following the elevated transactivation of PUMA and PERP, A276S illustrated higher induction of apoptosis than wild-type p53, as measured through 7-AAD staining, and morphological signs of apoptosis in T47D cells (with mutated and mislocalized endogenous p53). A276S caused higher cell death in HeLa cell lines (with unmutated endogenous p53) as well. These studies provide evidence that A276S can also execute higher apoptosis in a different cell line, regardless of the endogenous p53 status.

In this article, we demonstrated that our rationally designed A276S mutant p53 can up-regulate apoptotic target gene promoters and thereby cause higher cell death than wild-type p53. We have addressed A276 as a hot spot of p53. Interestingly, A276D mutation has been reported in some lung cancer patients. We also identified the select amino acid
properties required to fit and favorably interact with the 276th position. Even though a few improvised p53-variants have been discovered mostly in terms of protein stability,\(^3\)\(^\text{--}\)\(^5\) here we report a new p53 mutant that induces higher apoptosis by directly binding to a specific DNA sequence.

p53 is a top candidate for gene therapy for cancer. Of the three main functions of p53, apoptosis, cell cycle arrest, and DNA repair, we focused on improving the apoptotic function of p53. Our proposed p53 mutant, A276S, can specifically bind to and transactivate the promoters of selected apoptotic genes but not DNA repair or other off-target genes. In terms of therapeutic efficacy, A276S induced higher apoptosis than wild-type p53, yet maintained cell cycle arrest. These studies indicate the utility of using A276S mutant p53 for cancer therapy. Although the biological activity of A276S was explored in this article, further evidence on biochemical characterization is required to determine its biophysical properties such as protein stability. Further studies of A276S with other p53 promoters and in other cell lines could expand the enhanced death star properties of p53 and prove its utility as a potent anticancer agent, with greater efficacy than wild-type p53.

**ASSOCIATED CONTENT**

Additional figure depicts the result from Western Blot experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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