Stabilization of mutant p53 via alkylation of cysteines and effects on DNA binding

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Abstract: Oncogenic mutations inactivate the tumor suppressor p53 by lowering its stability or by weakening its binding to DNA. Alkylating agents that reactivate mutant p53 are currently being explored for cancer therapy. We have discovered ligands containing an α,β -unsaturated double bond, characteristic of Michael acceptors, that bind covalently to generic cysteine sites in the p53 core domain. They raised the melting temperature of the core domain of wild-type p53 and the hotspot mutants R175H, Y220C, G245S, R249S, and R282 by up to 3°C. Analysis of the relative reactivity of the cysteines in p53 by mass spectrometry found that C124 and C141 react first, followed by C135, C182, and C277, and eventually C176 and C275. Post-translational modifications of cysteines are known to be involved in regulation of other transcription factors. Modification of C277, which sits on the DNA-binding surface, may, for example, play a role in regulating p53 activity in cells in response to environmental cues. We found that the modifications progressively reduced DNA-binding activity of full-length p53. In light of these results, it is likely that the anticancer activity of the alkylating drugs works via a nontranscriptional activity of p53.

Keywords: protein; stability; alkylation; p53; DNA; binding

Introduction

The tumor suppressor protein p53 is inactivated by mutation in some 50% of human cancers. Some 30% of these mutations destabilize the core, DNA-binding domain, causing it to be temperature sensitive. The melting temperature (T_m) of the protein's core do-

main may even be lowered below that of body temperature, and it denatures irreversibly very rapidly.^{1–4} Most of the mutants have native structure at lower temperatures and, in principle, binding of a drug selectively to the native state of the protein could reverse the thermodynamic and kinetic denaturation consequences of these mutations.

The stabilization of mutant p53 by small molecules has received considerable attention as a potentially viable strategy to cancer therapy.^{5–8} The first approach was to find small molecules that bind non-covalently, either generically⁹ to sites present in wild-type protein or specifically to mutation-induced cavities,¹⁰ found from *in vitro* studies. The binding of compounds in those studies was characterized *in vitro* by biophysical and structural studies, and so, the observed activity in cells¹¹ has a biochemical basis. The modes of action of other molecules that induce apparent p53-reactivation in cellular assays remain more speculative.^{12–16} One compound of potential clinical importance, PRIMA-1, which upregulates p53 in cells, produces a hydrolysis product,

Abbreviations: ESI-nanospray MS, electrospray ionization-nanospray mass spectrometry; HSQC, heteronuclear single-quantum coherence; K_d , binding affinity; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDITOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; T_m , melting temperature; T-p53FL, full-length p53 with stabilized core domain; T-p53C, stabilized variant of p53 core domain; WT-p53C, wild-type p53 core domain.

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methylene quinuclidinone, that reacts with free sulfhydryl groups in proteins.¹⁷ Accordingly, there is the possibility that PRIMA-1 and related compounds enhance the stability of mutant p53 via covalent modification of the protein's cysteine residues. The cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin reportedly also stabilizes wild-type p53 in cells via covalent modification of cysteines in the protein's core domain.¹⁸

As part of studies to find small-molecule fragments that bind in a mutation-induced cavity in the p53 Y220C oncogenic mutant,^{19,20} we have now discovered molecules that bind covalently to the protein by alkylating cysteine residues. We measured the extent of modification of p53 by the fragments and identified the sites of modification in p53 and the order of their reactivity via mass spectrometry. Additionally, we determined the impact of modification on the stability of the protein and its DNA-binding activity. These results may shed light on potential cellular mechanisms that control p53 function in cells through modification of specific reactive residues.

Results and Discussion

Screening of small molecule library

Fragment-like molecules that bind to T-p53C-Y220C were identified via screening of the protein against an in-house compound library as described previously.¹⁹ Binding was measured by a high-throughput fluorimetric thermal denaturation assay. The hits obtained via the initial screening were then assayed individually by $^{15}\text{N}/^1\text{H}$ HSQC as a means to validate binding and, moreover, to map the residues on the surface of T-p53C-Y220C that are involved in interactions with the ligands.

Of the lead molecules identified, two in particular, 3-benzoylacrylic acid (1) and its fluorinated derivative (*E*)-4-(4-fluorophenyl)-4-oxobut-2-enoic acid (2) were of considerable interest based on their stabilizing effects and location of their target binding sites on T-p53C-Y220C [Fig. 1(A)]. In thermal denaturation scanning fluorimetry assays, the molecules both raised the apparent T_m of T-p53C-Y220C by nearly 2°C [Fig. 1(B)]. Even more interesting, however, is that both molecules appear to bind at a site that is remote from the mutation-induced crevice. In $^{15}\text{N}/^1\text{H}$ HSQC spectra of T-p53C-Y220C in complex with 1 and with 2, residues L114, H115, T123, Q136, C277, R282, and R280 were most significantly perturbed [Fig. 1(C)]. These residues correspond to a site on the opposite side of the protein, close to the DNA-binding interface. All previously reported molecules that bind p53-Y220C bind selectively to the vicinity of the mutated cysteine.^{10,19} Accordingly, it was presumed likely that these molecules could bind to a generic site on wild-type p53 and thus represent the basic scaffold for developing a drug that could reverse the effects of

Figure 1. Identification of the binding of 1 and 2 to T-p53C-Y220C. A: Chemical structures of molecules 1 and 2. B: Differential scanning fluorimetry trace of T-p53C-Y220C in absence (solid lines) and presence (dashed lines) of 1 (1 mM). Scans for three independent analyses with and without 1 are shown. C: Overlay of $^{15}\text{N}/^1\text{H}$ HSQC spectra of T-p53C-Y220C (green), T-p53C-Y220C with 1 (1 mM; purple), and T-p53C-Y220C with 2 (1 mM; red).

other cancer-related mutations in p53 that destabilize the protein. Considering that, collectively, the fraction of mutations that inactivate p53 by lowering the protein's melting temperature totals approximately 30–40%, the development of such a drug has considerable implications.

To substantiate the notion that molecules 1 and 2 bind to a generic site on p53, we assayed the core domain of wild-type p53 (WT-p53C) by $^{15}\text{N}/^1\text{H}$ HSQC in the presence of 1 and 2, separately. The resulting spectra confirmed binding of both molecules (Fig. 2).

Figure 2. Overlay of $^{15}\text{N}/^1\text{H}$ HSQC spectra of WT-p53C-Y220C (green), WT-p53C-Y220C with 1 (1 mM; purple), and WT-p53C-Y220C with 2 (1 mM; red).

Significant chemical shift perturbations were observed on binding in the same region of the protein that coincides with the target-binding site of 1 and 2 on T-p53C-Y220C. Subsequent to this, the binding of molecule 1 to other highly destabilized mutants of p53 was also studied. Specifically, the effect of 1 on the T_m of p53 core domain containing the mutations R249S, G245S, R282W, and R175H was determined by thermal denaturation scanning fluorimetry. Analysis of the resulting melting curves of the p53 mutants found that the T_m of each was markedly increased by 1, with T_m shifts ranging from 1.6–3.0°C for the different mutants (Fig. 3). The increase in the apparent T_m of p53 core domain increased with increasing concentration of 2 [Fig. 3(B)]. These data supports the premise that molecules 1 and 2 can bind and stabilize p53 in a mutation-independent manner.

Determination of binding mode of lead molecules to p53-Y220C core domain

To determine the detailed binding mode of the compounds, we performed crystallographic studies as previously described by soaking compound 1 into crystals of T-p53C-Y220C and collecting an X-ray data set to 1.75-Å resolution. Inspection of the electron density after initial refinement revealed unexpected electron density protruding from several Cys side chains, indicating varying degrees of covalent modification by the compound. The most pronounced difference density was observed for Cys277 although the density was not sufficient to model unambiguously the modification. Weak additional difference density at the sulfur atoms was also observed for C182 and C229, whereas C124, C135, C141, C220, and C275 did not show any sign of additional electron density. There were also

indications of small structural perturbations at the zinc-binding site. These observations suggested that molecule 1, and in all likelihood 2 as well, react specifically with thiol groups in the protein.

On the basis of consideration of the chemical structures of molecules 1 and 2, we hypothesized that both molecules, which contain an α,β -unsaturated double bond, are potential Michael acceptors. As such, the nucleophilic sulfhydryl group of cysteines in proteins may add across the α,β -unsaturated double bond via a classical 1,4-addition reaction. To test this hypothesis, we assayed the chemical reactivity of several structurally related

Figure 3. Stabilization of WT-p53C and p53 structural mutants T-p53C-Y220C, T-p53C-R249S, T-p53C-G245S, T-p53C-R282W, and T-p53C-R175H by 1. A: The increase in T_m of the respective proteins in the presence of 1 (1 mM) was measured by thermal denaturation scanning fluorimetry. B: The T_m of WT-p53C versus concentration of 1 for reactions incubated for the same period of time.

Figure 4. Binding of derivatives of 1 with and without an α,β -unsaturated double bond was assayed by thermal denaturation scanning fluorimetry. A: Chemical structures of derivatives of 1. B: Stabilization of T-p53C-Y220C and WT-p53C by derivative compounds (1 mM assay concentration) as measured by thermal denaturation scanning fluorimetry.

molecules with and without an α,β -unsaturated double bond toward T-p53C-Y220C as well as WT-p53C (Fig. 4). Thermal denaturation scanning fluorimetry found that the T_m of T-p53C-Y220C and WT-p53C was increased by between 0.9 and 2.2°C by molecules 6 ((E)-4-oxo-4-(p-tolyl)but-2-enoic acid), 8 ((E)-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid), and 9 ((E)-4-(4-ethoxyphenyl)-4-oxobut-2-enoic acid), which differ from 1 and 2 only by the substituent group on the benzyl ring. Conversely, as anticipated, molecules 3 (4-phenylbutanoic acid), 4 (4-oxo-4-phenylbutanoic acid), and 5 (2-amino-4-phenylbutanoic acid), which lack an α,β -unsaturated double bond, did not stabilize T-p53C-Y220C or WT-p53C. The effect of molecules 7 ((E)-4-(4-tert-butylphenyl)-4-oxobut-2-enoic

acid) and 10 ((E)-4-(4-cyclohexylphenyl)-4-oxobut-2-enoic acid), which also possess the requisite α,β -unsaturated double bond, could not be measured due to high background fluorescence (SYPRO orange likely associates with the highly hydrophobic moieties attached to the benzyl ring in the molecules, causing the exogenous dye to fluoresce strongly).

That only derivatives of 1 and 2 that contain an α,β -unsaturated double bond react with T-p53C-Y220C and WT-p53C confirms these molecules react via Michael addition with p53. Once anchored to p53, the pendant groups in 1 and 2 may interact non-covalently with neighboring residues, which have stabilizing effects on the protein. The strength of these interactions influence the degree to which the protein is stabilized. The interactions involving the pendant groups in 1 and 2 likely mimic non-covalent interactions formed by the binding of other fragments and small molecules that stabilize p53. This effect presumably is similar to that observed by the covalent attachment of polymers such as polyethylene glycol that also can lead to a dramatic increase in thermal stability.²¹ Incubation of WT-p53C with increasing concentrations of 1 and 2 would in theory lead to greater alkylation and thus more stabilizing non-covalent interactions (Fig 3B).

Our results show the direct link between the covalent modification of cysteines in p53 and increased p53 stability. This link provides insight into the potential mechanism of mutant p53 reactivation by the previously identified compounds MIRA-1, STIMA-3, and PRIMA-1. MIRA-1, which contains a thiol-reactive maleimide group, and STIMA-3, a quinazolinone derivative, are, like 1 and 2, potential Michael acceptors that can react with mutant p53 directly.^{13,16} PRIMA-1 was shown to covalently react with thiol groups in p53 on hydrolytic activation to methylene quinuclidinone, which also is a Michael acceptor.¹⁷ These molecules could rescue the function of destabilized mutant p53 by reacting with specific cysteines in the protein in its native state, which thermodynamically traps the protein in its active conformation. However, such compounds are presumably of limited clinical potential unless the reaction specificity of these compounds for mutant p53 over other proteins in cells can be enhanced.

Characterization of the reactivity of cysteines in p53

The results of crystallographic studies and thermal shift assays with derivatives of 1 and 2 establish that 1 and 2 react with T-p53C-Y220C and WT-p53C covalently. Figure 5 shows the location of cysteines in the structure of T-p53C-Y220C. To determine the extent of p53 modification with these compounds, the number of attached molecules of 1 using WT-p53C was analyzed by ESI-nanospray MS. The

Figure 5. Location of cysteines in the structure of T-p53C-Y220C. A: Ribbon diagram of T-p53C-Y220C (PDB code 2J1X).²⁰ Cysteine residues are highlighted as stick models. They are color-coded according to their reactivity with compound 1 in solution as determined by mass spectrometry: high reactivity (green), low reactivity (blue), and no reactivity under the experimental conditions (red). Residues 182 and 277 adopt alternative conformations in the crystal structure. B: Stereo view of the C124, C135, C141 cluster, suggesting that modification of these residues proceeds via a sequential mechanism with conformational changes through which the buried residues C135 and C141 become accessible. The figure was prepared using PyMOL (<http://www.pymol.org>).

Figure 6. Characterization of the extent of modification of WT-p53C by 1 via ESI-nanospray mass spectrometry. The molar ratio (0:1, 20:1, 50:1, 100:1) of compound-to-protein (50 μ M) employed in the modification reaction is indicated. The protein with one adduct does not have a single specifically labelled cysteine but is a mixture of singly labelled species. Similarly, the protein with two adducts does not have two specifically labelled species but is a mixture of doubly labelled molecules, etc.

Figure 7. Characterization of the extent of modification of T-p53FL by 1 via ESI-nanospray mass spectrometry. The molar ratio (0:1, 100:1, 150:1, 200:1, 250:1) of compound-to-protein (50 μ M) employed in the modification reaction is indicated. The protein with one adduct does not have a single specifically labelled cysteine but is a mixture of singly labelled species. Similarly, the protein with two adducts does not have two specifically labelled species but is a mixture of doubly labelled molecules, etc.

spectra of intact WT-p53C when reacted with 1 at molar ratios of compound-to-protein of 20:1, 50:1, and 100:1 show multiple peaks that correspond to a heterogeneous mixture of protein species that vary in number of modifications (Fig. 6). The offset between peaks is 176 Da, which corresponds to the size of 1. When reacted with 20:1 and 50:1 molar ratios of compound-to-protein, as many as two molecules of 1 were attached to WT-p53C. A third protein species to which three molecules of 1 were conjugated was

apparent on modification with the 100:1 molar ratio of compound-to-protein. The extent of WT-p53C modification, which approached 100% (i.e., no residual unmodified protein at m/z 24752) with the 100:1 ratio, increased with increasing ratios of compound-to-protein. This was evident by the apparent shift in distribution in the number of attached molecules of 1 per protein molecule. Of note, the molar ratio of compound-to-protein in the initial fragment screen from which 1 was identified was 70:1. Moreover, all of the

Table I. Solvent Accessibility and Theoretical pKa Values of Cysteine Side Chains in T-p53C-Y220C

Residue	Solvent-accessible surface (Å ²) ^a	pK _a ^b
Cys124	10.6	13.8
Cys135	0.1	39.1
Cys141	0.1	24.5
Cys176	3.9	10.1
Cys182	21.9	11.7
Cys220	9.7	11.8
Cys229	14.9	12.7
Cys238	0.2	24.6
Cys242	9.3	14.5
Cys275	5.1	20.0
Cys277	19.4	8.3

^a Solvent accessibility of cysteine side chains in the structure of T-p53C-Y220C (PDB entry 2J1X, chain A)²⁰ were calculated using the ProtSA server (<http://webapps.bifi.es/protsa/protSA.html>).²²

^b Theoretical pKa values of cysteines in the structure of T-p53C-Y220C were calculated using the H++ server (<http://biophysics.cs.vt.edu/H++/index.php>), which employs a standard continuum electrostatics methodology.²³

protected via coordination with an essential zinc atom [Fig. 5(A)]. Modification of the zinc ligand C176 was observed only at very high concentrations of 1. The high susceptibility of C124, C135, and C141 to alkylation by 1 was unexpected given that these residues are largely buried. However, the sulfur of C124 becomes accessible on simple rotation of the side chain, and modification of C124 presumably alters the local structure, facilitating subsequent modification of the adjacent C135 and C141 [Fig. 5(B)]. The cysteine thiol of C124 has a moderate pKa (13.8) relative to the other thiol groups in the native protein structure. It is interesting to note that there was no indication of modification of these cysteines in our initial crystallographic studies when soaking crystals of the Y220C mutant with 1. It appears that under these conditions C124 is inert to alkylation because of restricted accessibility and reduced structural plasticity of this site in the crystal lattice. Of the other cysteines that are alkylated at intermediate-to-high ratios of compound-to-protein, C182 and C277 are highly solvent accessible, whereas the side chain of C275 is partly buried and was accordingly less reactive. The predicted pKa (8.3) of the thiol of C277 is particularly low in the native structure, which makes it highly susceptible to modification. Given the high nucleophilicity and solvent accessibility of the sulfur atom, it is, at first sight, surprising that C220 is not modified at all. A plausible explanation for this is that the highly hydrophobic nature of the surrounding residues in the mutational cleft inhibits the relatively polar fragment from reaching C220. However, it is conceivable that a drug specific for C220 may be designed by incorporating a reactive Michael acceptor group to a less polar scaffold, such as PhiKan083 or one of the reported fragments, that binds within the mutational cleft.^{10,19}

Analysis of the structural implications of site-specific modifications of cysteines in p53 is potentially of im-

portance in understanding how p53 function is regulated in cells. In cellular assays, the transcriptional activity of p53 is abolished by oxidation of cysteines as well as the presence of alkylating agents.^{18,24–27} The modification of C275 and C277 are of particular interest from a functional standpoint. Alkylation of these sites would be expected to abrogate the DNA-binding activity of p53 since C275 and C277 sit at the DNA-protein interface. Consistent with this, Kim *et al.*¹⁸ recently showed that the transcriptional activity of p53 in cellular assays is abolished by selective attachment of the naturally occurring lipid 15-deoxy- $\Delta^{12,14}$ -prostaglandin to C277. The covalent modification of C277 by the prostaglandin, which also prolonged the longevity of p53 in cells, likewise occurs via Michael addition. Thus, modification of p53 at C275 and C277 enables a “switch” for controlling the biological activity as well as cellular degradation of p53. It is significantly more difficult to predict if the modification of C124, which is close to the N-terminus, C141, and C182 is important for p53 regulation given that modification of these sites does not have any obvious structural implications on p53 activity. Modification of C182 may have an effect on the self-complementary core domain-core domain interface that is formed on binding of two core domains of p53 to a consensus DNA half-site, which involves a pair of salt bridges between Glu180 and Arg181.^{6,28} This potentially makes this cysteine also an important site for controlling the biological activity of p53. Cysteine modification has been implicated in the regulation of other transcription factors including nuclear factor-kB, nuclear factor-E2 related factor 2, and activating protein-1.²⁹

Impact of BAA modification on DNA-binding activity of full-length p53

The binding of T-p53FL modified with differing extents of 1 to a consensus DNA sequence was measured by fluorescence anisotropy. Alkylation of p53 led to marked reduction in DNA-binding (Table II). The apparent binding affinity of T-p53FL modified with a 200:1 and 250:1 molar ratio of compound to 50- μ M protein was 3.5- and 5-fold weaker, respectively, than that of T-p53FL (Table II). The binding curves were determined by adding increasing concentrations of p53 as a titrant to labeled DNA, and so the apparent dissociation constants depends on the fraction of each species

Table II. Impact of Covalent Attachment of Compound 1 on the DNA-Binding Activity of T-p53FL^a

Concentration of 1 (mM)	K _d (nM)
0	86 $\pm$ 14
10	300 $\pm$ 40
12.5	445 $\pm$ 120

^a Fifty micromolar protein solution was incubated as described in text. The K_d is the concentration of p53 (monomers) required for 50% saturation of DNA. It is an ensemble value of the values for all modified species.

of modified p53 present and its dissociation constant. On the basis of analysis of relative peak intensities in Figure 7, we estimated the 200:1 modified sample to contain ~11% residual unmodified protein in addition to 41% protein with one modification, 46% protein with two modifications, and 2% protein with three modifications. The 250:1 modified sample contained an estimated 2% residual unmodified protein, 33% protein with one modification, 62% protein with two modifications, and 2% protein with three modifications. The observed values of K_d were too high to be accounted for by binding only to residual unmodified p53. The reduced binding affinity of modified T-p53FL resulted from weaker binding on alkylation, most likely of C275 and C277. This further points toward the modification of these reactive cysteines as a possible mechanism for p53 regulation in cells.

Conclusions

In conclusion, we have identified through fragment screening novel ligands that thermodynamically stabilize mutant and wild-type p53 alike via reacting with cysteines in the respective proteins. These ligands share a common α,β -unsaturated double bond to which the thiol group of cysteines can add across through a 1,4-addition mechanism. In addition to showing the direct link between covalent modification and increased p53 stability, we identified the nature and, specifically, the order of the reactivity of the cysteines in p53. The high reactivity of specific cysteine thiol groups in p53 are likely important for the regulation of p53 activity and degradation pathways in cells in response to environmental cues. Our analysis of the reactivity of the cysteines in p53 found C277, which sits on the DNA-binding surface and thus whose modification presumably would destroy p53 activity and may alter cellular levels of p53 by impairing its specific degradation pathways, to be among the most reactive. Further implicating the role of covalent modification of specific cysteines in p53 regulation, DNA-binding experiments found the activity of p53 was severely reduced on alkylation. Ultimately, these findings, in addition to shedding light on potential cellular mechanisms that control p53 function in cells, provide insight into how previously identified thiol-reactive compounds may rescue p53 function. It is clear that modification of certain cysteines, in particular C277, abolishes transcriptional functions of p53 but could have an effect on p53's nontranscriptional functions via modulation of cellular protein levels.

Materials and Methods

Materials

The fragment library used for screening p53-Y220C consisted of fragments from Chembridge (San Diego,

CA), Life Chemicals (Kiev, Ukraine), and Maybridge (Cornwall, UK). The fluorescent dye SYPRO orange was purchased from Invitrogen (Carlsbad, CA). The alkylating agent 3-benzoylacrylic acid was obtained from Aldrich (St. Louis, MO). Derivatives of 3-benzoylacrylic acid were purchased from Aldrich, Maybridge, Acros Organics (Pittsburgh, PA), Enamine (Kiev, Ukraine), and Alfa-Aesar (Heysham, UK). Crystallography reagents were from Hampton Research (Aliso Viejo, CA). The fluorescent-labeled p53 recognition sequence Alexa488-GGACATGTCCGGACATGTCC³⁰ used in DNA-binding experiments was synthesized in-house.

Protein expression and purification

For screening of the fragment library, a stable variant (T-p53C-Y220C; residues 94–312) of the p53 core domain^{31,32} with the Y220C mutation was used.²⁰ The protein was expressed with an N-terminal His6 lipoyl tag and purified as described previously.¹⁰ For ¹⁵N/¹H 2-dimensional NMR experiments, the protein was expressed in M9 minimal media with ¹⁵N-labeled ammonium chloride as the only nitrogen source. For crystallization, a tag free form of the protein was produced using the same procedure. Wild-type p53 core domain (WT-p53C) and full-length p53 containing the stabilized core domain (T-p53FL) were similarly produced for fragment binding and DNA binding assays, respectively.

Fragment screening

The fragment library was screened against T-p53C-Y220C via thermal differential scanning fluorimetry. High-resolution melting of T-p53C-Y220C (15 μ M) in the presence of individual fragments (1 mM final concentration) in buffer (25 mM potassium phosphate, 150 mM sodium chloride, 5 mM dithiothreitol, pH 7.5) containing the fluorescent dye SYPRO orange (diluted 1000-fold) and dimethyl sulfoxide [5% (v/v) final concentration] was monitored using a Corbett (Mortlake, Australia) Rotor-Gene 6000 real-time PCR thermocycler. The assay temperature was ramped from 30 to 50°C at a rate of 0.1°C/s. Fluorescence was recorded continuously using excitation and emission filters of 460 and 510 nm, respectively. The T_m of T-p53C-Y220C in the presence and absence of ligand was determined as a function of the first derivative of the fluorescence-melting curve. A minimum T_m shift of 0.5°C was used as the critical threshold for the determination of fragment hits. Samples were run in triplicates and the resulting T_m values averaged.

Characterization of fragment binding by NMR

Fragment binding to T-p53C-Y220C and WT-p53C was confirmed by ¹⁵N/¹H heteronuclear single-quantum coherence (HSQC) NMR. Briefly, fragment (1 mM final concentration) dissolved in neat dimethylsulfoxide-*d*6 [5% (v/v) final concentration] was added to protein

(75 μM) in buffer (25 mM potassium phosphate, 150 mM sodium chloride, 5 mM dithiothreitol, pH 7.2). All $^{15}\text{N}/^1\text{H}$ fast-HSQC spectra were acquired at 20°C on a Bruker Avance-800 spectrometer (800 MHz ^1H frequency) using a 5-mm inverse cryogenic probe. Raw data comprised 1024 points in f_2 and 64 complex points in f_1 , with spectral widths of 13.8 ppm and 34 ppm in f_2 and f_1 , respectively. The target-binding site of the fragments was analyzed by chemical shift mapping using 0.1 ppm as a critical threshold for significant perturbations in ^{15}N or ^1H resonances.³³

Crystallographic determination of fragment binding mode

Crystals of T-p53C-Y220C were grown via the sitting drop vapor diffusion method as described previously.²⁰ On growing, the crystals were soaked in a 25 mM solution of 3-benzoylacrylic acid in cryo buffer (19% polyethylene glycol 4000, 20% glycerol, 10 mM sodium phosphate, pH 7.2, 100 mM HEPES, pH 7.2, 150 mM potassium chloride, dithiothreitol 10 mM) for 50 min and flash frozen in liquid nitrogen immediately after. A complete X-ray dataset was collected at 100 K at beamline I02 of the Diamond Light Source (Oxford, UK) and subsequently processed with MOSFLM³⁴ and SCALA.³⁵ The structure was solved by rigid body refinement using the structure of ligand-free mutant as a search model using PHENIX³⁶ and further refined using PHENIX and COOT.³⁷

Characterization of modified core domain and full-length p53 by mass spectrometry

For modification of WT-p53C and T-p53FL, protein (50 μM on dilution) was added to buffer (25 mM potassium phosphate, 150 mM sodium chloride, 5 mM dithiothreitol, pH 7.5) containing varying amounts of compound and dimethyl sulfoxide [5% (v/v) final concentration]. The absolute concentration of compound in the reaction solution was 0, 1, 2.5, 5, 7.5, 10, or 12.5 mM. The reaction solution was incubated for 2 h at 4°C on a rotating shaker. Residual excess compound (i.e., free compound in solution) was then removed via overnight dialysis in reaction buffer at 4°C using a 3500 or 10000 MWCO Promega (Madison, WI) dialysis cassette. Modified proteins are subsequently referred to in terms of the relative molar ratio of compound-to-protein employed in the modification reaction (generally 50 μM protein).

The mass of intact p53-modified proteins was determined by electrospray (ESI) MS on an API QSTAR Pulsar instrument (AB MDS Sciex; Foster City, CA) fitted with a nanoelectrospray source (MDS Protana; Odense, Denmark). Protein solutions were desalted using Millipore (Bedford, MA) C4 zip-tips prior to mass analysis.

Solutions of modified p53 proteins were diluted to 5 μM with 50 mM ammonium bicarbonate (pH 7.8) and digested with porcine trypsin (Promega; Madison, WI; 1:100 enzyme:protein; w:w) overnight

at 37°C. The digest was terminated by the addition of trifluoroacetic acid to a final concentration of 1% (v/v), and the peptides were desalted using a Millipore C18 zip-tip prior to analysis by MS analysis. For matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS the desalted peptide sample was mixed 1:2 (v/v) with a 10 mg/mL solution of α -cyano-4-hydroxycinnamic acid and spotted onto the MALDI target using the dried droplet method. MALDI-TOF data was acquired on a Bruker Daltonics (Bremen, Germany) Ultraflex III in reflector ion mode from m/z 500–5000 to detect all of the p53 peptides. Data-dependent liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out by nanoflow reverse phase liquid chromatography [using a U3000 from Dionex (Paris, France)] coupled online to a Thermo Scientific (Waltham, MA) LTQ-Orbitrap XL mass spectrometer. Briefly, the LC separation was performed using a Dionex C18 PepMap column (75- μm ID $\times$ 150 mm; 3- μm bead size), and the peptides were eluted using a linear gradient from 5% solvent B to 50% solvent B over 40 min at a flow rate of 200 nL/min (solvent A: 2% acetonitrile in 0.1% formic acid; solvent B: 90% acetonitrile in 0.1% formic acid). A cycle of one full FT scan mass spectrum (350–1800 m/z , resolution of 60,000 at m/z 400) was followed by 6 data-dependent MS/MS acquired in the linear ion trap. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2). Mascot was set up to search the p53 sequence assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 ppm. Oxidation of methionine, acetylation of the N-terminus, and modification of cysteine with compound were specified in Mascot as variable modifications. Scaffold (version Scaffold 3.00.03, Proteome Software) was used to validate MS/MS-based peptide identifications and MS/MS spectra of the cysteine containing peptides were manually checked.

DNA-binding activity assay

The DNA-binding activity of T-p53FL modified with differing amounts of compounds was measured by fluorescence anisotropy. The protein was similarly modified as described for MS analysis above. Protein (1.25 μM titrant concentration) was titrated into a cuvette in a thermostatted holder containing fluorescent-labeled double-stranded DNA (20 nM initial concentration) in buffer [25 mM sodium phosphate, 5 mM dithiothreitol, 10% (v/v) glycerol], pH 7.2, I = 225 mM) with constant stirring using a Hamilton (Bonaduz, Switzerland) Microlab 500 titrator. Fluorescence anisotropy of the labeled DNA was continuously monitored using a Varian (Palo Alto, CA) Cary Eclipse fluorescence spectrometer at 25°C with excitation and emission filters of 480 and 530 nm, respectively. Data were fit to the Hill equation from

which the dissociation constant (K_d = concentration of protein for 50% binding) for the protein–DNA complex was extracted.

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References

- Bullock AN, Henckel J, DeDecker BS, Johnson CM, Nikolova PV, Proctor MR, Lane DP, Fersht AR (1997) Thermodynamic stability of wild-type and mutant p53 core domain. *Proc Natl Acad Sci USA* 94:14338–14342.
- Bullock AN, Henckel J, Fersht AR (2000) Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. *Oncogene* 19:1245–1256.
- Joerger AC, Fersht AR (2007) Structure-function-rescue: the diverse nature of common p53 cancer mutants. *Oncogene* 26:2226–2242.
- Joerger AC, Fersht AR (2008) Structural biology of the tumor suppressor p53. *Annu Rev Biochem* 77:557–582.
- Brown CJ, Lain S, Verma CS, Fersht AR, Lane DP (2009) Awakening guardian angels: drugging the p53 pathway. *Nat Rev Cancer* 9:862–873.
- Joerger AC, Fersht AR (2010) The tumor suppressor p53: from structures to drug discovery. *Cold Spring Harb Perspect Biol* 2:a000919.
- Selivanova G (2010) Therapeutic targeting of p53 by small molecules. *Semin Cancer Biol* 20:46–56.
- Wiman KG Pharmacological reactivation of mutant p53: from protein structure to the cancer patient. *Oncogene* 29:4245–4252.
- Friedler A, Hansson LO, Veprintsev DB, Freund SM, Rippin TM, Nikolova PV, Proctor MR, Rudiger S, Fersht AR (2002) A peptide that binds and stabilizes p53 core domain: chaperone strategy for rescue of oncogenic mutants. *Proc Natl Acad Sci USA* 99:937–942.
- Boeckler FM, Joerger AC, Jaggi G, Rutherford TJ, Veprintsev DB, Fersht AR (2008) Targeted rescue of a destabilized mutant of p53 by an in silico screened drug. *Proc Natl Acad Sci USA* 105:10360–10365.
- Issaeva N, Friedler A, Bozko P, Wiman KG, Fersht AR, Selivanova G (2003) Rescue of mutants of the tumor suppressor p53 in cancer cells by a designed peptide. *Proc Natl Acad Sci USA* 100:13303–13307.
- Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, Bergman J, Wiman KG, Selivanova G (2002) Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 8:282–288.
- Bykov VJ, Issaeva N, Zache N, Shilov A, Hultcrantz M, Bergman J, Selivanova G, Wiman KG (2005) Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs. *J Biol Chem* 280:30384–30391.
- Foster BA, Coffey HA, Morin MJ, Rastinejad F (1999) Pharmacological rescue of mutant p53 conformation and function. *Science* 286:2507–2510.
- North S, Pluquet O, Maurici D, El-Ghissassi F, Hainaut P (2002) Restoration of wild-type conformation and activity of a temperature-sensitive mutant of p53 (p53(V272M)) by the cytoprotective aminothiols WR1065 in the esophageal cancer cell line TE-1. *Mol Carcinog* 33:181–188.
- Zache N, Lambert JM, Wiman KG, Bykov VJ (2008) PRIMA-1MET inhibits growth of mouse tumors carrying mutant p53. *Cell Oncol* 30:411–418.
- Lambert JM, Gorzov P, Veprintsev DB, Soderqvist M, Segerback D, Bergman J, Fersht AR, Hainaut P, Wiman KG, Bykov VJ (2009) PRIMA-1 reactivates mutant p53 by covalent binding to the core domain. *Cancer Cell* 15:376–388.
- Kim DH, Kim EH, Na HK, Sun Y, Surh YJ (2010) 15-Deoxy-delta(12,14)-prostaglandin J(2) stabilizes, but functionally inactivates p53 by binding to the cysteine 277 residue. *Oncogene* 29:2560–2576.
- Basse N, Kaar JL, Settanni G, Joerger AC, Rutherford TJ, Fersht AR (2010) Toward the rational design of p53-stabilizing drugs: probing the surface of the oncogenic Y220C mutant. *Chem Biol* 17:46–56.
- Joerger AC, Ang HC, Fersht AR (2006) Structural basis for understanding oncogenic p53 mutations and designing rescue drugs. *Proc Natl Acad Sci USA* 103:15056–15061.
- Rodriguez-Martinez JA, Sola RJ, Castillo B, Cintron-Colon HR, Rivera-Rivera I, Barletta G, Griebenow K (2008) Stabilization of alpha-chymotrypsin upon PEGylation correlates with reduced structural dynamics. *Biotechnol Bioeng* 101:1142–1149.
- Estrada J, Bernado P, Blackledge M, Sancho J (2009) ProtSA: a web application for calculating sequence specific protein solvent accessibilities in the unfolded ensemble. *BMC Bioinformatics* 10:104.
- Gordon JC, Myers JB, Foltz T, Shoja V, Heath LS, Onufriev A (2005) H++: a server for estimating pKas and adding missing hydrogens to macromolecules. *Nucleic Acids Res* 33:W368–W371.
- Fojta M, Kubiarova T, Vojtesek B, Palecek E (1999) Effect of p53 protein redox states on binding to supercoiled and linear DNA. *J Biol Chem* 274:25749–25755.
- Hainaut P, Milner J (1993) Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res* 53:4469–4473.
- Rainwater R, Parks D, Anderson ME, Tegtmeyer P, Mann K (1995) Role of cysteine residues in regulation of p53 function. *Mol Cell Biol* 15:3892–3903.
- Stoner CS, Pearson GD, Koc A, Merwin JR, Lopez NI, Merrill GF (2009) Effect of thioredoxin deletion and p53 cysteine replacement on human p53 activity in wild-type and thioredoxin reductase null yeast. *Biochemistry* 48:9156–9169.
- Kitayner M, Rozenberg H, Rohs R, Suad O, Rabinovich D, Honig B, Shakked Z (2010) Diversity in DNA recognition by p53 revealed by crystal structures with Hoogsteen base pairs. *Nat Struct Mol Biol* 17:423–429.
- Na HK, Surh YJ (2006) Transcriptional regulation via cysteine thiol modification: a novel molecular strategy for chemoprevention and cytoprotection. *Mol Carcinog* 45:368–380.
- Veprintsev DB, Fersht AR (2008) Algorithm for prediction of tumour suppressor p53 affinity for binding sites in DNA. *Nucleic Acids Res* 36:1589–1598.
- Joerger AC, Allen MD, Fersht AR (2004) Crystal structure of a superstable mutant of human p53 core domain. Insights into the mechanism of rescuing oncogenic mutations. *J Biol Chem* 279:1291–1296.
- Nikolova PV, Henckel J, Lane DP, Fersht AR (1998) Semirational design of active tumor suppressor p53

- DNA binding domain with enhanced stability. *Proc Natl Acad Sci USA* 95:14675–14680.
33. Hajduk PJ, Dinges J, Miknis GF, Merlock M, Middleton T, Kempf DJ, Egan DA, Walter KA, Robins TS, Shuker SB, Holzman TF, Fesik SW (1997) NMR-based discovery of lead inhibitors that block DNA binding of the human papillomavirus E2 protein. *J Med Chem* 40:3144–3150.
 34. Leslie AG (1999) Integration of macromolecular diffraction data. *Acta Crystallogr D Biol Crystallogr* 55: 1696–1702.
 35. Evans P (2006) Scaling and assessment of data quality. *Acta Crystallogr D Biol Crystallogr* 62:72–82.
 36. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC (2002) PHENIX: building new software for automated crystallographic structure determination. *Acta Cryst D* 58:1948–1954.
 37. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486–501.
 38. Friedler A, Veprintsev DB, Hansson LO, Fersht AR (2003) Kinetic instability of p53 core domain mutants: implications for rescue by small molecules. *J Biol Chem* 278:24108–24112.