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Stapled Peptides with Improved Potency and Specificity That Activate p53

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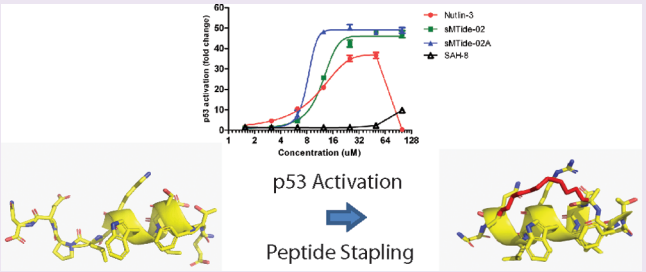
Supporting Information

ABSTRACT: By using a phage display derived peptide as an initial template, compounds have been developed that are highly specific against Mdm2/Mdm4. These compounds exhibit greater potency in p53 activation and protein−protein interaction assays than a compound derived from the p53 wild-type sequence. Unlike Nutlin, a small molecule inhibitor of Mdm2/Mdm4, the phage derived compounds can arrest cells resistant to p53 induced apoptosis over a wide concentration range without cellular toxicity, suggesting they are highly suitable for cyclotherapy.



Many protein−protein interactions involve a contiguous section of protein that forms an interfacial α-helix when bound. This conformation can be stabilized by a chemical method known as stapling, which consists of a covalent linkage connecting adjacent turns of the helix (Figure 1a). Stapling peptides can increase their affinity by reducing the entropic cost of binding, increase their *in vivo* half-life by improving their proteolytic stability, and, most significantly, allow their cellular uptake.[1−4] This technology has the potential to allow protein interactions of biological and medical interest to be functionally interrogated and assessed for their suitability for therapeutic development. The data presented here validates stapled peptides as a new class of macrocyclic compounds, which are capable of interacting with intracellular targets with high affinity.

Inhibition of the p53:Mdm2 interaction is an attractive therapeutic target. [5] Agonists of this interaction can activate the p53 response by blocking the two inhibitory activities of Mdm2, namely, its occlusion of the N-terminal p53 trans-activation domain and its targeting of p53 for ubiquitination and proteasomal degradation. Such molecules can reactivate p53 function in p53 wild-type tumor cells.[6] In a second application, called cyclotherapy, their ability to induce a reversible cell cycle arrest in normal proliferating cells can selectively protect these tissues from cytotoxic chemotherapeutics and ionizing radiation, thus enabling the treatment of p53 null or p53 mutant tumors with fewer side effects.[6,7]

Several classes of molecules that inhibit this interaction have been developed (e.g., Nutlin[5] and MI-219[8]). They mimic the conserved residues from a region of the p53 N-terminal that are essential for the interaction with the N-terminal p53 binding domain of Mdm2.[6] This region forms an α-helix upon binding, enabling the three conserved residues of the Mdm2 binding motif (FXXXWXXL) to optimally embed into the hydrophobic binding groove located on Mdm2 and the homologous Mdm4 protein.[9,10] Bernal et al.[11] have reported a stapled peptide (SAH-8) derived from wild-type p53 that binds to Mdm2/Mdm4 (Figure 1a) and activates the p53 response in cells. The cocrystal structure of SAH-8 in complex with Mdm2 has been solved confirming simulations by our own group showing the staple making extra contacts with Mdm2 (Figure 1b).[12,13]

As the wild-type p53 peptide (E^{1}TFSDLWKLLP^{11}E) has a low affinity for Mdm2/Mdm4[10,11] and comes from a region of p53 that interacts with many other proteins, we explored the effects of stapling a peptide derived from phage selection experiments. Phage display and rational design methods have been used to isolate linear peptides that bind Mdm2 with high affinity.[10,14,15] The most avid of these published peptides, described by Pazgier et al.,[10,16] was used as the template for this

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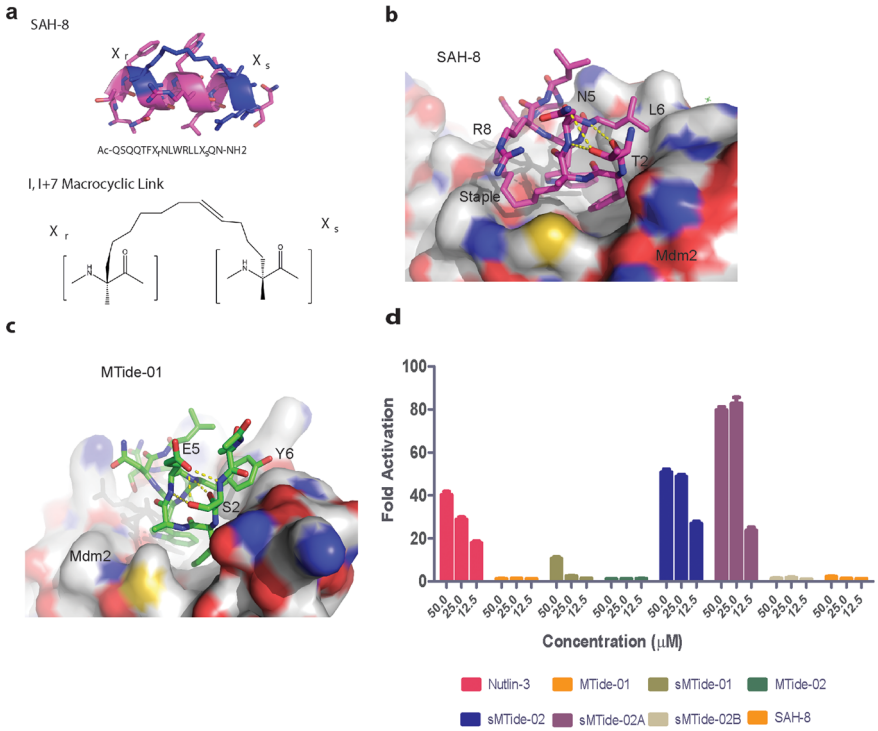

Figure 1. Design and biological screening of sMTide analogues. (a) The Mdm2/4 interacting peptides were stapled via an I, I + 7 linkage between positions 4 and 11. The upper panel shows the linkage (blue) across two turns of the p53 peptide helix (SAH-8), and the lower panel shows the structure of the staple between the two α-methyl, α-alkenyl amino acids (*R* and *S* optical isomers, respectively) incorporated into the peptide sequence. (b) Crystal structure of SAH-8 in complex with Mdm2 (3V3B) showing the staple interacting with the protein surface and the side-chain of T2 interacting with the backbone amide and side chain of N5. (c) A representative snapshot from a computer simulation of MTide-01 in complex with Mdm2 highlighting the hydrogen bond network around E5 and S2. E5 also interacts with the amide backbone of S2 further stabilizing the N-terminal of the bound helix. (d) Linear and stapled peptides were tested for biological activity in a T22 p53 reporter cell line.

Table 1. Apparent K_ds and Equivalent IC_{50}s Determined by Competitive Fluorescence Anisotropy Titrations; Presence of I, I+7 Staple Indicated by X; Biological Activity Determined by T22 p53 Reporter Assay

		Mdm2		Mdm4		T22 assay (p53 activity)
ligand	primary sequence	K_d (nM)	IC_{50} (μM)	K_d (nM)	IC_{50} (μM)	fold activation (25 μM)
MTide-01	Ac-^{1}TSFAEYWNLLS^{11}-NH2	46.34 ± 6.89	1.10 ± 0.10	33.16 ± 4.62	0.55 ± 0.04	1.4 ± 0.01
sMTide-01	Ac-^{1}TSFX$_r$EYWNLLX$_s^{11}$-NH2	86.99 ± 0.02	1.80 ± 0.10	118.3 ± 0.04	1.57 ± 0.22	2.62 ± 2.51
MTide-02	Ac-^{1}TSFAEYWALLS^{11}-NH2	28.04 ± 1.38	0.75 ± 0.02	16.33 ± 2.00	0.29 ± 0.02	1.23 ± 0.03
sMTide-02	Ac-^{1}TSFX$_r$EYWALLX$_s^{11}$-NH2	34.35 ± 2.03	0.94 ± 0.04	45.73 ± 7.65	0.66 ± 0.06	48.63 ± 1.85
sMTide-02A	Ac-^{1}TSFX$_r$EY(L-6-Cl)WALLX$_s^{11}$-NH2	6.76 ± 2.11	0.32 ± 0.02	1360 ± 600	3.49 ± 0.63	82.67 ± 2.90
sMTide-02B	Ac-^{1}TSFX$_r$EY(D-6-Cl)WALLX$_s^{11}$-NH2	88.16 ± 7.20	1.95 ± 0.41	2160.73 ± 1000	11.6 ± 2.2	1.67 ± 0.33
SAH-8	Ac-QSQ^{1}QTFX$_r$NLWRLLX$_s^{11}$QN-NH2	50.21 ± 5.53	1.11 ± 0.09	14.03 ± 1.85	0.26 ± 0.03	1.34 ± 0.05

study. P12 was removed from the original Pazgier sequence as computer simulations demonstrated that induction of the helix by the staple would prevent the proline from packing optimally against the Mdm4 surface. Also, P12 is not observed in the electron density map in the crystal structure of the Mdm2:peptide complex and is not critical for binding to Mdm2.[10] This resulting peptide termed MTide-01 (T^1SFAEYWNLLS^{11}) interacts strongly and specifically with Mdm2/Mdm4 (Table 1). The staple was incorporated across positions 4 and 11 in MTide-01 to create the derivative sMTide-01.

The mechanism responsible for the improved binding of the Pazgier peptide and MTide-01 in comparison to the wild-type sequence,[10] as supported by our simulation data, is the optimization of an intramolecular hydrogen bond network that stabilizes its helical conformation when bound to Mdm2/Mdm4, which is centered on S2 and E5 (Figure 1c). Also, S11 located at the C-terminal of the peptide further enhances its helicity.[12] Position 11 also corresponds to where the staple is tethered in sMTide-01, thereby replacing the helix inducing property of S11. Alanine scanning[16] identified N8 as being detrimental to Mdm2/4 binding primarily through disruption of the bound helix as Asn is rarely located within solvent exposed central regions of α-helices. We therefore replaced Asn8 with Ala8 to create the sequence termed MTide-02 and the stapled derivative sMTide-02 (Table 1).

In comparison to their unstapled counterparts, sMTide-01/02 exhibited no significant decrease in their K_ds, while, respectively, showing a ~2-fold to 4-fold increase in their K_ds against Mdm4 (Table 1). sMTide-01 displayed little biological activity in a T22 derived p53 reporter cell line (Figure 1d).[17] In contrast, sMTide-02 induced the strongest p53 transcriptional response that we have ever seen in this assay, which has been used to screen more than 300,000 compounds.[18,19] The SAH-8

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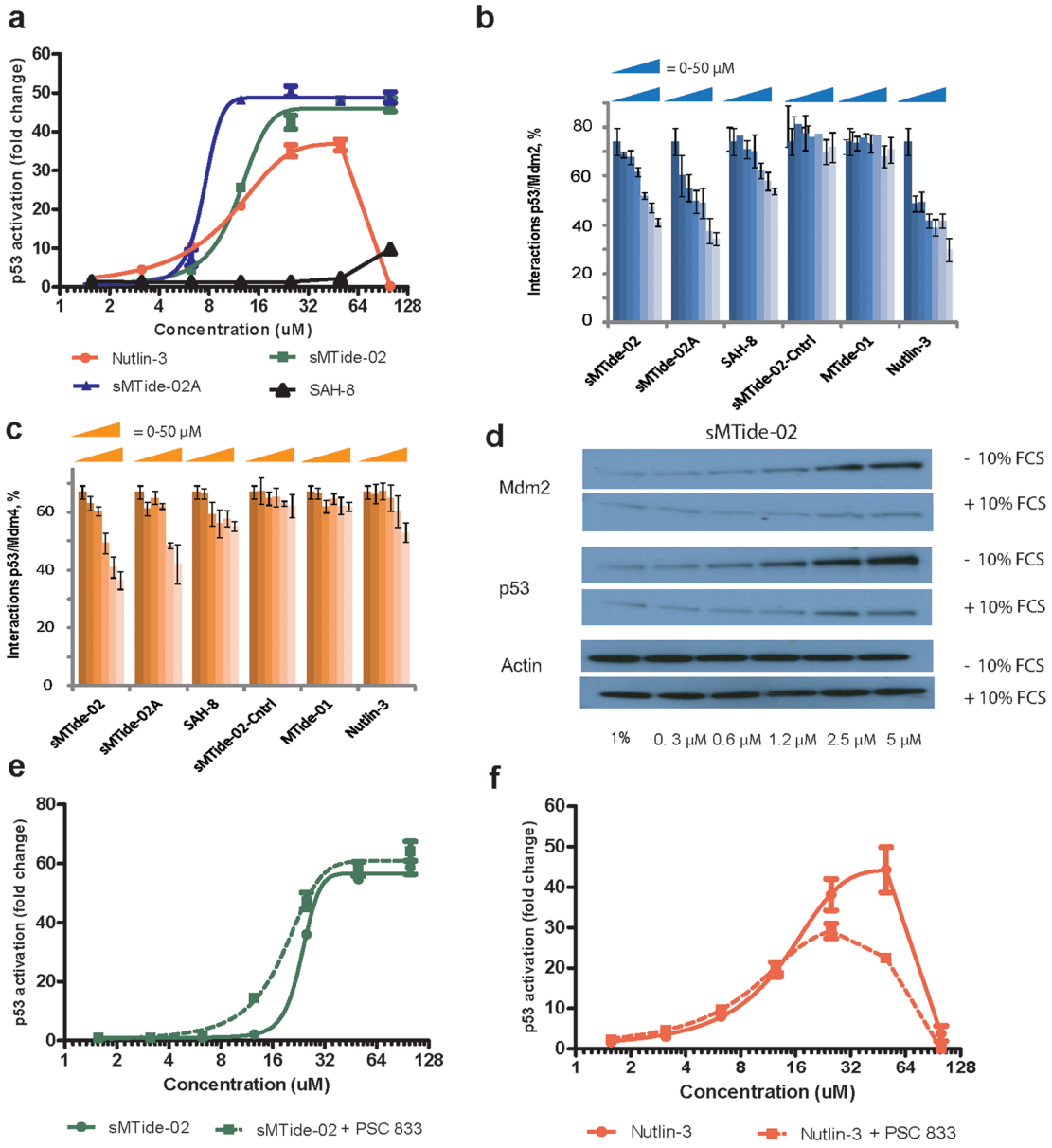

Figure 2. Bioactive potency of sMTide peptides. (a) Dose response curves showing titrations of Nutlin, sMTide-02, sMTide-02A, and SAH-8 in the T22 p53 transcriptional activity assay. (b,c) Titration of stapled peptides, control peptides, and Nutlin into the F2H assay modeling the interaction of p53 with Mdm2 (b) or Mdm4 (c) in living BHK cells. Graph bars show means of normalized interaction values (in %) ± SEM from three independent experiments. (d) Western blot analysis of HCT-116 p53 +/+ cells treated with a 2-fold dilution series of sMTide-02A peptide for 6 h with or without fetal calf serum (FCS). (e,f) Titrations of Nutlin and sMTide-02 into the T22 p53 reporter assay with and without 20 μM of the PGP efflux inhibitor PSC-883, respectively.

peptide was also synthesized, and it K_ds were determined to be 50.21 ± 5.53 and 14.03 ± 1.85 nM against Mdm2 and Mdm4, respectively. These compare well with the published values of 55 ± 11 nM[11] and 2.3 ± 0.2 nM. [20] The placement of the staple in the SAH-8 sequence causes an increase in affinity of the peptide against Mdm2 and Mdm4 (compared to 410 ± 19[11] and 646 ± 26 nM,[10] respectively, for the wild-type peptide) in contrast to the MTide based sequences (see Table 1). In addition, it also shows a preference for Mdm4 over Mdm2. When tested in the T22 p53 reporter assay, SAH-8 had negligible activity compared to sMTide-02 (Figure 1d).

The addition of a chlorine atom at the C6 position of W7 is known to improve the potency of peptides that interact with Mdm2/4.[21,22] Two stapled peptide analogues of sMTide-02 were synthesized containing either the L (termed sMTide-02A) or D (termed sMTide-02B) optical isomers of the 6-Cl modified tryptophan. The L-isomer bound Mdm2 with an improved K_d of 6.76 ± 2.11 nM, but its affinity for Mdm4 was significantly attenuated, akin to Nutlin (Table 1 and Supplementary Figure S1). The D-isomer showed negligible activity in the T22 assay despite interacting with Mdm2 with an apparent K_d of 88.16 ± 7.20 nM indicating either poor cell permeability or an inability to disrupt the pre-existing p53:Mdm2 interactions within the cell. Its K_d with Mdm4 was also attenuated (Table 1). The L-isomer sMTide-02A showed much higher fold induction of p53 transcriptional activity than the unmodified sMTide-02 in the T22 assay (Figure 1d).

A further set of stapled peptides was generated to explore the effect of position 8 on their potency and biological activity (Supplementary Figure S2 and Table S1). These peptides

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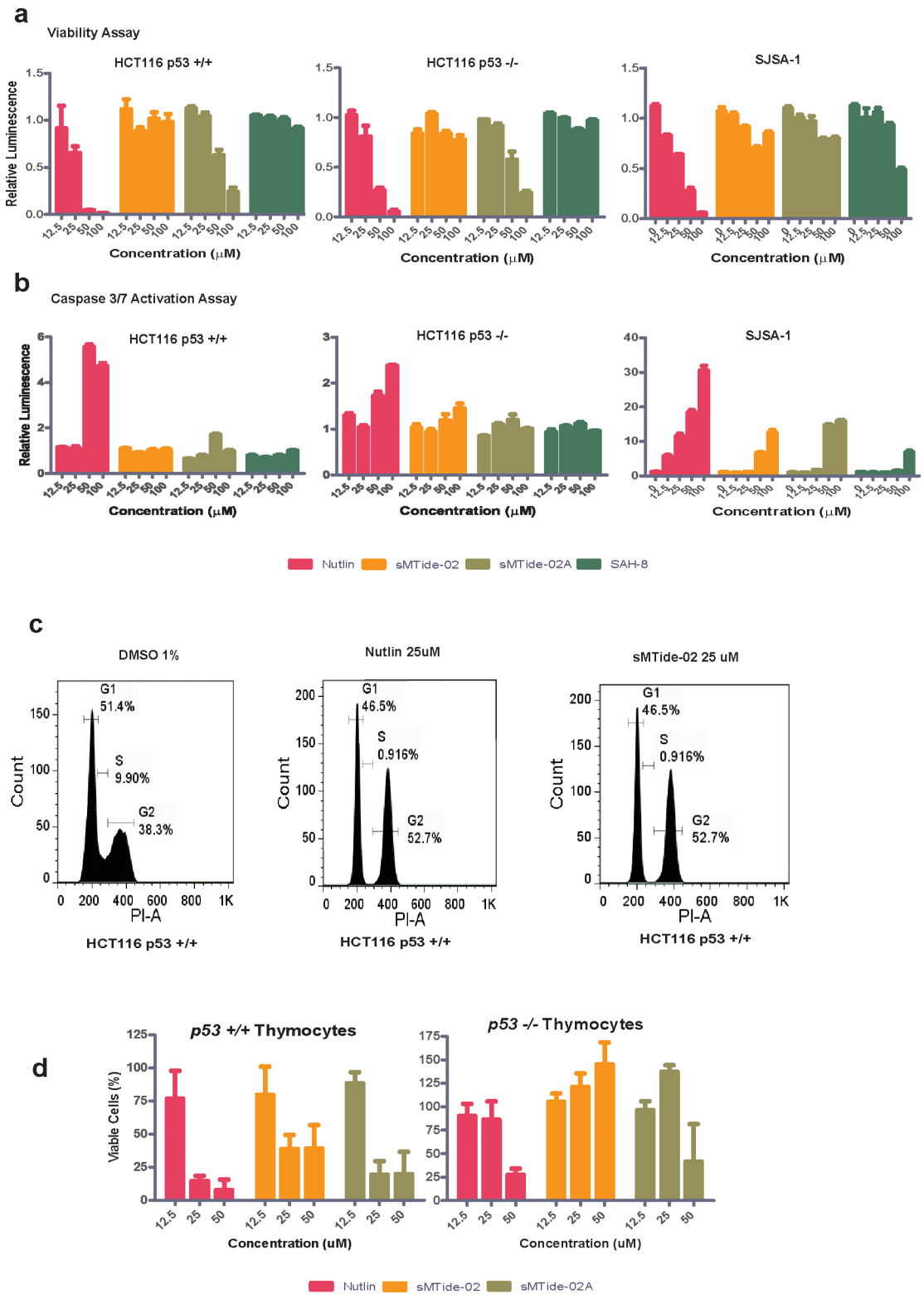

Figure 3. Characterization of cellular response. (a) Cell viability dose responses as indicated by intracellular ATP levels (Cell-Titer-Glow viability assay, Promega) and (b) caspase 3/7 activity dose responses (Caspase-Glo 3/7 assay, Promega) of various cell lines against Nutlin and the stapled peptides at 24 h. (c) Flow cytometry histograms showing the cell cycle profiles of propidium iodide stained HCT116 p53 +/+ cells in response to treatments with the stapled peptides and Nutlin for 24 h. (d) Thymocytes from either wild-type or p53 knockout mice were isolated and treated with Nutlin or sMTide-02/02A peptides. Thymocytes were stained with annexin V and PI, and the percentage of viable cells (negative for PI and annexin V) after 24 h were plotted against compound concentration.

exhibited a wide range of K_ds that did not correlate with their activity in the p53 reporter assay. For example, if Ile8 or Phe8 are introduced, the binding of the stapled peptide to Mdm2/4 is attenuated compared to sMTide-01, but their biological activity in the p53 reporter assay is improved. These results show that the context of the sequence within which the staple is

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utilized ultimately determines its effectiveness and that there is a complex relationship between the affinity of the compound for its target protein and its biological activity. Intriguingly, the small addition of a hydroxyl, i.e., Phe8 vs Tyr8, (sMTide-07/06, respectively) has little effect on the interaction between these two peptides and Mdm2/4. However, it does diminish the ability of sMTide-06 to induce p53 compared to sMTide-07 (Supplementary Table S1). Understanding how these types of changes influence the ability of the stapled peptide to enter cells efficiently in relation to their physiochemical properties is the subject of further study and is not addressed in this work.

Titrations of p53 activating compounds into the T22 assay typically produce a bell-shaped curve in which high concentrations of compound produce lower levels of reporter protein as a result of cell toxicity. Is this toxicity p53 dependent or p53 independent? Remarkably, while Nutlin induces a typical bell-shaped curve, the two peptides (sMTide-02 and sMTide-02A) show a sigmoidal curve with a plateau over a large dose range with much higher levels of reporter protein production, indicating that these compounds lack cell toxicity, despite their ability to activate p53 function to high levels (Figure 2a). However, both stapled peptides at low concentrations induce less p53 activity than Nutlin, perhaps indicating that the dynamics of cell entry for these molecules are different.

To further investigate the mechanism of action of the stapled peptides in live cells, a fully reversible cellular protein−protein interaction assay was used. The fluorescent 2-hybrid (F2H) assay[23] (ChromoTek GmbH) is a microscopy-assisted method developed to analyze the disruption of the p53 interaction with either Mdm2 or Mdm4 within the nucleus of BHK cells (Figure 2b,c). sMTide-02 and sMTide-02A were both shown to dissociate Mdm2 from p53 with the former showing greater potency against Mdm4. Interestingly, the SAH-8 peptide shows poor activity in this assay and limited ability to inhibit either Mdm2/4. The selectivity of Nutlin toward Mdm2 is exquisitely demonstrated by the F2H assay. Live cell observations revealed that both the stapled peptides and Nutlin dissociated Mdm2/Mdm4 from p53 within 60 min (Supplementary Figure S3).

The low activity of SAH-8 in the protein interaction assay and the T22 assay prompted us to examine the precise conditions used to measure the biological activity of stapled peptides. We noted that Bernal et al.[11] removed serum from the media prior to the addition of SAH-8 to cells, while our studies were conducted in the presence of fetal calf serum. The effect of serum on the activity of the stapled peptides was dramatic with sMTide-02A, sMTide-02, and SAH-8 showing significantly improved potency in the absence of serum (Figure 2d and Supplementary Figure S4). However, the F2H assay showed a much smaller difference in the ability of the compounds to disrupt the complexes of Mdm2/4 with p53 in the presence or absence of serum, suggesting that serum removal sensitizes the p53 pathway to stimuli rather than limit peptide entry into cells.

To further probe the mechanism of action of the stapled peptides, HCT-116 p53 +/+ cells were pretreated with the PGP inhibitor PSC-883, a nonimmunosuppressive cyclosporine A analogue. The titration of sMTide-02 with PSC-883 in the reporter assay significantly improved the sensitivity of the p53 response (Figure 2e), but the titration with Nutlin yielded no such improvement (Figure 2f). Interestingly, the toxicity observed when Nutlin is titrated alone (the decrease in p53 dependent reporter gene product due to cell death) occurs at lower concentrations in the presence of PSC-883. Inhibition of the PGP efflux pump therefore seems to be potentiating the presumed p53 independent cellular toxicity of Nutlin, while it has no effect on p53 induction as this is already efficiently activated.

The effects of Nutlin and the improved specificity of sMTide-02/02A were further studied in isogenic cell lines that were either wild-type or null for p53 (Figure 3a,b). sMTide-02 and sMTide-02A caused no significant decrease in the viability of either HCT-116 p53 +/+ or HCT-116 p53 −/− cells and induced negligible caspase 3/7 activity. Nutlin and surprisingly SAH-8 exhibited distinctly different characteristics to the sMTide-02/02A peptides with both compounds decreasing cell viability at high concentrations in both cell lines. Interestingly, Nutlin induced caspase 3/7 activity in the two cell lines tested, with higher fold levels observed in HCT-116 p53 +/+ cells. Analysis of the cell cycle distribution of cells possessing wild-type p53 indicated that the sMTide-02/02A peptides caused G_1/G_2 arrest at both low (Figure 3c) and high concentrations (Supplementary Figure S5), while Nutlin at low concentrations also caused G_1/G_2 arrest (Figure 3c) but at higher concentrations caused substantial cell death. SAH-8 stabilizes p53 at much higher concentrations than Nutlin and sMTide-02/02A. This correlates with the observed decreases in cellular viability for the p53 wild-type and null cells (Figure 3a). These results indicate that (a) sMTide-02 and sMTide-02A induce a specific p53 response when they inhibit Mdm2/4 repression in HCT-116 p53 +/+ cells but do not cause the cells to undergo p53 dependent apoptosis; (b) Nutlin, at high concentrations, induces apoptosis in a p53 independent manner indicating an off target effect; and (c) SAH-8 causes substantial cell death, irrespective of the presence of the wild-type p53 gene.

To ensure that the sMTide-02/02A peptides could still induce apoptosis, they were titrated into the SJSA-1 cell line, which is sensitive to p53 dependent cell death. Both peptides induced caspase 3/7 activity at substantially higher concentrations than Nutlin, which suggests that the off-target effect of Nutlin plays a role in the efficient induction of apoptosis in SJSA-1 cells (Figure 3a,b). Primary thymocytes are also known to be sensitive to p53 dependent radiation induced apoptosis. Thymocytes were isolated from p53 wild-type and p53 deficient mice[24] and then treated with Nutlin or the sMTide-02/02A peptides. In this assay, the two peptides induced cell death in a p53 dependent manner. Notably high doses of Nutlin and, to a certain extent, sMTide-02A caused apoptosis in p53 null thymocytes but sMTide-02 did not. This indicates an extraordinary degree of p53 dependent specificity (Figure 3d), confirming that, in this cell type, at least, p53 activation is sufficient to induce cell death.

These results suggest that, when p53 is activated by inhibition of Mdm2 repression, an additional cellular signal is often required to induce efficient apoptosis of cells. With Nutlin, the signal that causes the larger increase of caspase 3/7 activity in cells possessing wild-type p53 may reside in the nonspecific effects observed in the cells null for p53. Indeed, Nutlin has been reported to interact with the BCL protein family.[25] Such a mechanism implies that, for p53 reactivation to be a suitable therapy for cancer, an additional treatment (e.g., inhibition of proteins like MCL-1, BCL-2, ATM, or MET)[26] may be needed. Compared to sMTide-02/02A, SAH-8 leads to cell death in both isogenic HCT-116 cell lines, demonstrating the difference in origin of their respective peptide sequences. The p53 sequence, from which SAH-8 is derived, is also known

to interact with other proteins (e.g., p300 and TAFIIb) including Mdm2/4, which may explain the p53 independent cell death phenotype in p53 null cells and its toxicity to p53 wild-type cells. In contrast, the phage-derived MTide sequence was selected to interact specifically with Mdm2/4 and also only encompasses the length of sequence required for binding to Mdm2/4.

The phage derived sMTide-02/02A compounds are more specific and potent in their mode of biological action than SAH-8. These properties make the sMTide-02/02A peptides highly suitable for validating drug targets and even in parsing well understood small molecule therapies into specific and nonspecific contributions. However, it still remains to be understood how stapled peptides enter cells and the precise parameters that affect this property. sMTide-02/02A are also suitable candidates for dual therapy treatments in conjunction with an apoptosis promoting compound and may be very useful in cyclotherapy approaches where cellular arrest with low toxicity is required. It will prove, like Nutlin, to be a powerful tool for the p53 community.

METHODS

Complete methods for protein purification, K_d determination, computational modeling, and cellular activity assays, as well as the intracellular colocalization protein−protein interaction assay (F2H), are included in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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