Protein–Protein Interactions

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A Chemical Inhibitor of the Skp2/p300 Interaction that Promotes p53-Mediated Apoptosis

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Abstract: *Skp2 is thought to have two critical roles in tumorigenesis. As part of the SCF^{Skp2} ubiquitin ligase, Skp2 drives the cell cycle by mediating the degradation of cell cycle proteins. Besides the proteolytic activity, Skp2 also blocks p53-mediated apoptosis by outcompeting p53 for binding p300. Herein, we exploit the Skp2/p300 interaction as a new target for Skp2 inhibition. An affinity-based high-throughput screen of a combinatorial cyclic peptoid library identified an inhibitor that binds to Skp2 and interferes with the Skp2/p300 interaction. We show that antagonism of the Skp2/p300 interaction by the inhibitor leads to p300-mediated p53 acetylation, resulting in p53-mediated apoptosis in cancer cells, without affecting Skp2 proteolytic activity. Our results suggest that inhibition of the Skp2/p300 interaction has a great potential as a new anticancer strategy, and our Skp2 inhibitor can be developed as a chemical probe to delineate Skp2 non-proteolytic function in tumorigenesis.*

The SCF^{Skp2} is an E3 ubiquitin ligase complex that executes a tightly regulated degradation of a set of protein substrates, including key cell cycle proteins such as p27 and p21.[1] Skp2 is an F-box protein of the SCF^{Skp2} complex and responsible for recruiting and ubiquitylating substrate proteins. The poly-ubiquitinated proteins are then recognized and degraded by the 26S proteasome (Figure 1a). Several lines of evidence show that Skp2 is overexpressed in many cancers and promotes tumor progression and metastasis by downregulating tumor suppressor proteins.[1a,b,2] Thus, inhibition of oncogenic Skp2 function has emerged as a promising anticancer strategy. Indeed, several inhibitors have recently been reported, which include an inhibitor of the Skp1/Skp2 interaction,[3] an inhibitor of the interaction between p27 and Skp2,[4] an inhibitor of Skp2 incorporation,[5] and an inhibitor of the Skp2/Cks1 interaction.[6]

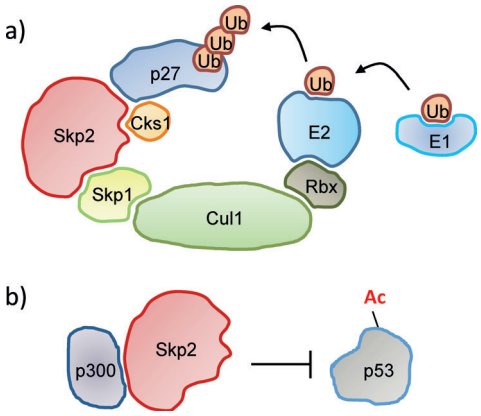

Figure 1. a) Skp2, as a component of the SCF^{Skp2} E3 ubiquitin ligase complex, promotes ubiquitin-mediated protein degradation. b) Skp2 blocks p300-mediated p53 acetylation by outcompeting p53 for binding p300.

In addition to its role in protein degradation (proteolytic activity), Skp2 is believed to have an alternative role in tumorigenesis. Recent studies have shown that Skp2 binds to p300, a transcriptional coactivator possessing acetyltransferase activity, and blocks its interaction with tumor suppressor protein p53, thereby preventing p300-mediated p53 acetylation.[7] Because acetylation is required for p53 stabilization and activation,[8] Skp2 acts as a negative regulator of p53-mediated transcription and apoptosis (Figure 1b). Notably, this activity is independent of ubiquitin-mediated proteolysis and is termed "non-proteolytic activity" of Skp2.[7a] Given that loss of tumor suppressor p53 function is implicated in cancer progression, and Skp2 is overexpressed in many aggressive cancers, inhibition of the Skp2/p300 protein–protein interaction (PPI) for re-activating p53 could be an attractive approach for cancer treatment. None of the reported Skp2 inhibitors[3–6] target the Skp2 non-proteolytic function mediated by the Skp2/p300 interaction. Herein, we report the first chemical inhibitor of the Skp2/p300 interaction. We demonstrate that the inhibitor promoted p300-mediated acetylation of p53 by antagonizing the Skp2/p300 interaction, thereby inducing p53-mediated apoptosis and cell growth inhibition in cancer cells.

In general, targeting PPIs with small drug-like molecules is a daunting task, mostly owing to the relatively large protein interfaces.[9] In this study, we employed macrocyclic peptoids as larger-sized molecules to effectively cover interfaces involved in PPIs. Cyclic peptoids are thought to have several important features as protein-capture agents.[10] They not only have conformational rigidity, but also retain the well-known

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advantages of peptoids, including good cell permeability and proteolytic stability.[11] As a result, macrocyclic peptoids could serve as an excellent source of protein-binding molecules. We recently developed facile solid-phase synthesis of various macrocyclic peptoids, and an efficient method for sequence determination of cyclic peptoids.[12] In this method, a cyclic peptoid on a single bead from a one-bead one-compound (OBOC) combinatorial library is converted into a linear peptoid by an oxidative ring-opening reaction, and the structure of the linearized peptoid can be revealed by MS/MS analysis. Therefore, our method enables preparation of a large OBOC library of cyclic peptoids with no need for encoding. To target Skp2, we synthesized a combinatorial library of triazine-bridged cyclic peptoids with a theoretical diversity of 161051 (11^5) by split-and-pool synthesis (Supporting Information, Figure S1).

To obtain ligands for Skp2 among the library molecules, we performed an affinity-based on-bead screen (Figure 2),

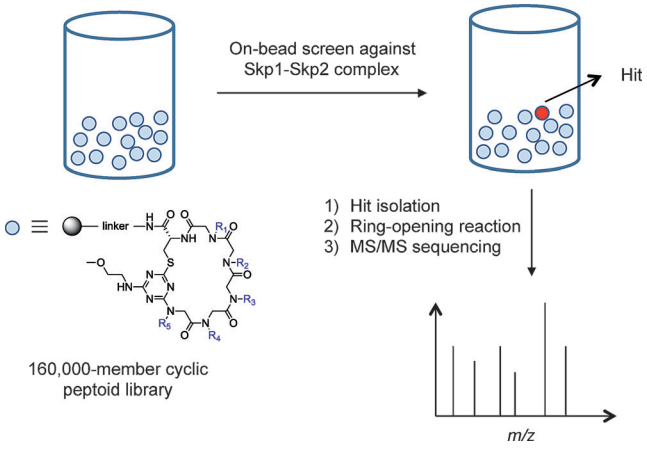

Figure 2. The on-bead screen of an OBOC combinatorial library of cyclic peptoids to identify molecules that directly bind to Skp1-Skp2.

which enables discovery of molecules capable of directly binding to a target protein.[13] After 3 rounds of screening the 160000-member library beads for their ability to bind Skp1ΔΔ-Skp2Δ, a deletion construct of human Skp1-Skp2 complex,[14] followed by sequence analysis (Figure S2), we identified M1 as a final hit compound (Figure 3a). The binding affinity of M1 was evaluated by fluorescence anisotropy assay. A fluorescently labeled derivative, M1-FL (Figure 3a), was synthesized and purified by HPLC (Figure S3). M1-FL exhibited binding to Skp1ΔΔ-Skp2Δ in a dose-dependent fashion with a K_D value of 3.85 μM, while N1-FL, a negative control (Figure 3a), did not, indicating that the fluorescein and backbone structure have minimum contributions to the binding affinity of M1 to Skp1ΔΔ-Skp2Δ (Figure 3b).

Because the Skp1-Skp2 dimeric complex was used for both on-bead screening and FP binding assays, it remained unclear whether M1 bound to Skp2 or Skp1. To determine the direct binding partner of M1, we employed a dihydroxyphenylalanine (DOPA)-mediated chemical cross-linking technology.[15] A biotinylated and DOPA-conjugated derivative M1-

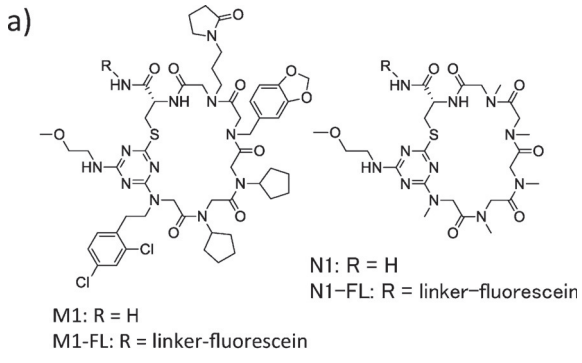

Figure 3. a) Structure of M1 and N1 and their fluorescein-labeled derivatives, M1-FL and N1-FL. b) M1 binds to Skp1-Skp2 complex with K_D value of 3.85 ± 0.53 μM, while N1 does not.

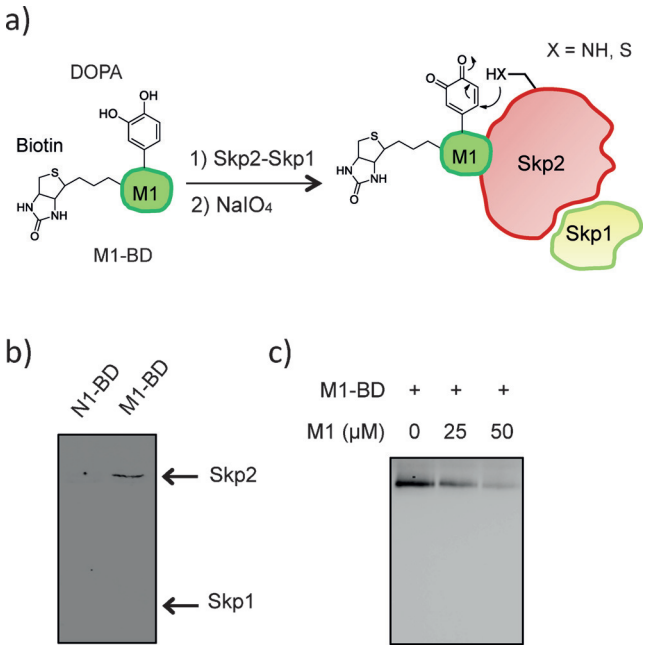

Figure 4. a) A depiction of chemical cross-linking. b) Cross-linking reaction between Skp1-Skp2 and M1-BD or N1-BD. c) Cross-linking reaction between Skp1-Skp2 and M1-BD in the presence of the indicated concentrations of M1.

BD (Figure 4a; Supporting Information, Figure S4) was incubated with purified Skp1ΔΔ-Skp2Δ for 10 min, followed by treating with $NaIO_4$ for 1 min. Subsequent gel electrophoresis and Western blotting with streptavidin-HRP

revealed the cross-linked product with a molecular mass of 36 kDa that apparently matched with Skp2, whereas no band corresponding to Skp1 was observed (Figure 4b). These data clearly show that M1 binds exclusively to Skp2, but not Skp1. The cross-linking reaction using N1-BD (a negative control) did not show any significant band, indicating that the cross-linking was attributed to the direct binding between Skp2 and M1 without effects from DOPA, biotin, or the linker. When M1 was added to the cross-linking reaction between M1-BD and Skp1-Skp2, the cross-linked product was reduced dose-dependently (Figure 4c), implying that M1-BD and M1 bind to the same binding site on Skp2.

Based on these in vitro assay results, including fluorescence anisotropy binding assays and cross-linking experiments, we initially anticipated that M1, as a direct Skp2-binding ligand, could serve as a functional inhibitor of Skp2-mediated proteolysis, thereby resulting in increased cellular levels of substrate proteins such as p27 and p21. To examine this point, HeLa cervix adenocarcinoma cells or SJSA-1 cells were treated with the indicated concentrations of M1, and then Western blotting assessed cellular levels of p27 and p21. To our surprise, we found that M1 elevated p21 levels in a dose-dependent manner, but had no significant effect on p27 levels (Figure 5a; Supporting Information, Figure S7). N1 did not exhibit any effect on cellular levels of p21 and p27 (Figure S7). Because p27 and p21 are the substrate proteins recognized by Skp2, both proteins should be upregulated if M1 inhibited the Skp2-mediated proteolysis similar to other Skp2 inhibitors.[3,4] To further examine whether, despite its binding to Skp2, M1 has no effect on the Skp2 proteolytic function, we performed in vitro competition assays where the complex of Skp1-Skp2/Cks1/fluorescently-labeled p27 peptide was incubated with increasing concentrations of M1, and then fluorescence anisotropy was monitored. Consistent with the cellular assays, no significant anisotropy change was observed, suggesting that M1 binds to a distinct surface area of Skp2 without affecting the proteolytic activity (Figure S8).

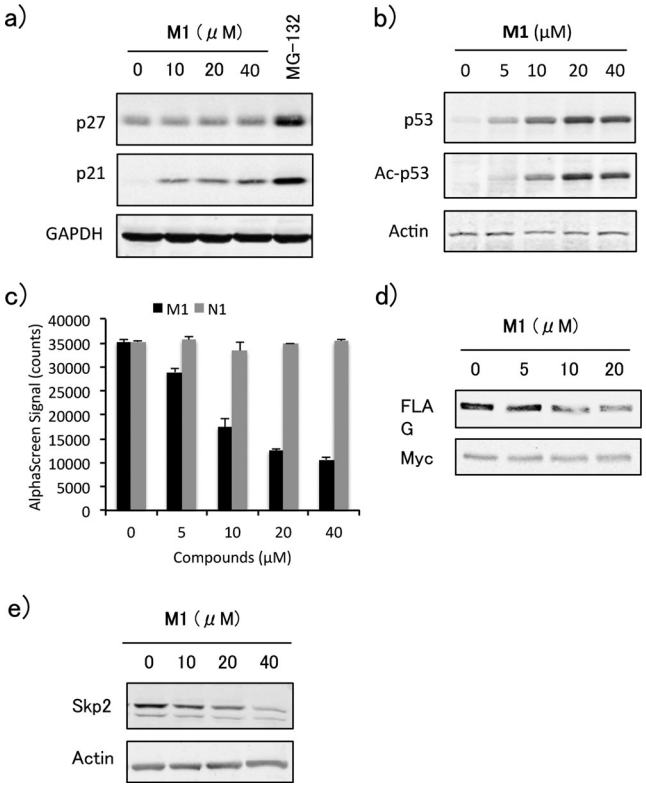

Figure 5. a) M1 up-regulates p21, but not p27, in HeLa cells. b) M1 increases cellular levels of p53 and acetylated p53 in ADR-stimulated HeLa cells. c) AlphaScreen assay showing that M1 antagonizes the Skp2/p300 interaction, whereas N1 has no effect. Error bars = standard deviation from three independent experiments. d) In vitro binding assay showing that M1 inhibits the interaction between FLAG-Skp2 and Myc-p300. e) M1 down-regulates Skp2 in HeLa cells.

From these intriguing findings, we speculated the possibility that M1 targets the p300-binding site of Skp2 rather than the substrate-binding site and thus blocks the Skp2/p300 interaction. Disrupting the interaction by M1 would liberate p300 from Skp2. Then, p300 can bind to p53 for acetylation, thereby rescuing the transactivation ability of p53.[7a] Given that p21 is a major transcription target of p53, it is likely that M1 increases cellular levels of p21 by stimulating p53-mediated transcription without changing p27 levels.[7a] To test this hypothesis, we assessed the effect of M1 on Skp2 non-proteolytic functions related to p53 acetylation and stabilization. HeLa or SJSA-1 cells were incubated with varying concentrations of M1 or N1 in the presence of 0.5 $\mu g\,mL^{-1}$ adriamycin (ADR). ADR is a DNA-damaging agent that increases basal levels of p53, which otherwise is at very low level. Given that Skp2 suppresses DNA damage-induced p53 activation,[7a] M1 would increase p53 levels if it compromises Skp2 activity. Whole cell lysates were analyzed by Western blots. Indeed, treatment of M1 led to significantly elevated levels of p53, as well as acetylated p53 (Figure 5b; Supporting Information, Figure S7), while N1 had no effect (Figure S7). These results suggest that M1 promotes p300-mediated p53 acetylation by blocking the Skp2/p300 interaction.

To examine if M1 disrupts the Skp2/p300 interaction, we used AlphaScreen (amplified luminescent proximity homogeneous assay screen), which is an efficient bead-based assay that detects PPI when two proteins are in close proximity through specific binding.[6] To the mixture of GST-Skp2 and His-tagged p300 (aa 1514–1922) containing the essential motif for binding to Skp2,[7a] varying concentrations of M1 or N1 were added and incubated for 1 h. After adding glutathione donor beads, and nickel chelate acceptor beads, the effect of the compounds on the Skp2/p300 interaction was evaluated by measuring energy transfer between the two beads. As seen in Figure 5c, M1 dose-dependently reduced the signal, demonstrating that M1 inhibited the interaction between Skp2 and p300. To further examine the antagonistic activity of M1 on the Skp2/p300 interaction, we performed in vitro binding assays using purified FLAG-tagged Skp2 and Myc-tagged p300. Western blot analysis exhibited that Skp2 bound to p300 was decreased by M1 (Figure 5d), supporting the idea that M1 antagonizes the Skp2/p300 interaction.

Because Skp2 stability is also known to be regulated by p300-mediated acetylation,[7b] disruption of the Skp2/p300 interaction by M1 would lead to a decrease in cellular levels of Skp2. We found that indeed M1 reduced Skp2 levels significantly in HeLa cells in a dose-dependent manner

(Figure 5e). These results further support the proposal that M1 inhibits the interaction between Skp2 and p300.

Given that p53 is a key player in apoptotic cell death through caspase activation, we next determined whether M1 promotes p53-mediated apoptosis. HeLa cells or WS-1 normal human fibroblast cells were treated with various concentrations of M1, and the apoptotic activity was evaluated using the Caspase-Glo 3/7 assay kit. As expected, M1 induced caspase activation in HeLa cells, presumably by promoting p53 stabilization, but had no effect in WS-1 cells (Figure 6a). These results indicate that M1 could be selectively toxic to malignant cells. To examine the selective cytotoxicity of M1, cell viability assays were performed using various cells. Cells were exposed to the indicated concentrations of M1, and cell viability was assessed by MTS cell proliferation assays. M1 suppressed malignant cell growth dose-dependently, whereas it had no significant effect on WS-1 cell viability (Figure 6b), which is in a good agreement with the apoptosis assay (Figure 6a). N1 did not exhibit an inhibitory effect on cell growth (Supporting Information, Figure S7). Overall, the data presented here demonstrate that M1 acts as an inhibitor of Skp2 non-proteolytic activity by disrupting the Skp2 interaction with p300, thereby inducing p300-mediated p53 stabilization and apoptosis. Further biological studies and structural modifications to improve its potency are underway.

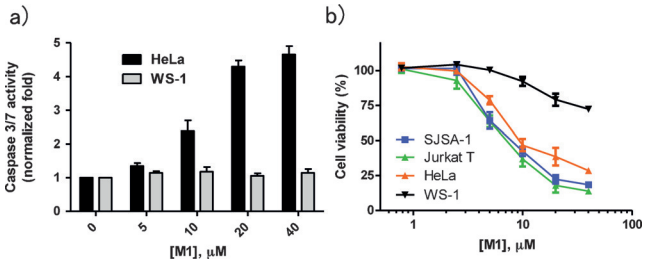

Figure 6. a) Apoptotic activity of M1 on HeLa and WS-1 cells analyzed by Caspase-Glo 3/7 assay kit. Error bars = standard deviation from two independent experiments. b) The effect of M1 on cell viability of various cells. Error bars = standard deviation from two independent experiments.

In summary, we have described the identification of M1 as a Skp2 inhibitor. To the best of our knowledge, this molecule represents the first example of a chemical inhibitor of the Skp2/p300 interaction. We showed that the inhibitor promotes p300-mediated p53 acetylation and stabilization, thereby inducing p53-mediated apoptosis and cell death in cancer cells. It is important to note that our Skp2 inhibitor is able to specifically inhibit non-proteolytic activity by disrupting the Skp2/p300 interaction without affecting Skp2 proteolytic activity. In contrast, biological methods such as gene knockout or RNA interference are limited in precisely understanding each role of Skp2 because they suppress both Skp2 proteolytic and non-proteolytic activities by deleting or downregulating Skp2. Thus, our Skp2 inhibitor holds great potential to be developed as a chemical probe to delineate Skp2 non-proteolytic function during tumorigenesis, and as a therapeutic candidate for the treatment of cancer.

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