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The development of inhibitors of intracellular protein–protein interactions (PPIs) is of great significance for drug discovery, but the generation of a cell-permeable molecule with high affinity to protein is challenging. Oligo(N-substituted glycines) (oligo-NSGs), referred to as peptoids, are attractive as potential intracellular PPI inhibitors owing to their high membrane permeability. However, their intrinsically flexible backbones make the rational design of inhibitors difficult. Here, we propose a peptoid-based rational approach to develop cell-permeable PPI inhibitors using oligo(N-substituted alanines) (oligo-NSAs). The rigid structures of oligo-NSAs enable independent optimization of each N-substituent to improve binding affinity and membrane permeability, while preserving the backbone shape. A molecule with optimized N-substituents inhibited a target PPI in cells, which demonstrated the utility of oligo-NSA as a reprogrammable template to develop intracellular PPI inhibitors.

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Peptoid-Based Reprogrammable Template for Cell-Permeable Inhibitors of Protein–Protein Interactions

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Abstract

The development of inhibitors of intracellular protein–protein interactions (PPIs) is of great significance for drug discovery, but the generation of a cell-permeable molecule with high affinity to protein is challenging. Oligo(N-substituted glycines) (oligo-NSGs), referred to as peptoids, are attractive as potential intracellular PPI inhibitors owing to their high membrane permeability. However, their intrinsically flexible backbones make the rational design of inhibitors difficult. Here, we propose a peptoid-based rational approach to develop cell-permeable PPI inhibitors using oligo(N-substituted alanines) (oligo-NSAs). The rigid structures of oligo-NSAs enable independent optimization of each N-substituent to improve binding affinity and membrane permeability, while preserving the backbone shape. A molecule with optimized N-substituents inhibited a target PPI in cells, which demonstrated the utility of oligo-NSA as a reprogrammable template to develop intracellular PPI inhibitors.
Introduction

Protein–protein interactions (PPIs) play fundamental roles in diverse biological processes and are involved in the pathogenesis of various diseases. Extensive efforts have been devoted to developing PPI inhibitors. However, it remains challenging to generate inhibitors of intracellular PPIs because it is difficult to produce a molecule having a large recognition surface area and high membrane permeability.

Peptidomimetic synthetic oligomers, such as β-peptides, peptoids, oligoureas, and γ-AApeptides, are attractive PPI inhibitor candidates because oligomers with desirable sequences can be prepared using well-established modular synthetic methods. Among them, peptoids, usually referring to oligo(N-substituted glycines) (oligo-NSGs, Fig. 1a), are advantageous in that diverse functional substituents can be introduced during oligomer synthesis using a submonomer synthetic method, and they have high membrane permeability owing to their N-substituted amide structure. Peptoids that inhibit intracellular PPIs, e.g., the interaction between Skp2 and p300, have been reported. However, the reported peptoids showed relatively weak binding to proteins and their $K_D$ values remain in the µM order. The reason for their low affinity has been considered to be the entropic penalty upon binding due to their intrinsically flexible backbone.

To overcome this limitation of oligo-NSGs, attempts have been made to realize conformationally constrained peptoids, for example, by the introduction of bulky N-substituents and head-to-tail macrocyclization. Recently, a peptoid that inhibits the interaction between β-catenin and T-cell factor 3 in cells was elegantly developed using conformation-constraining strategies. The peptoid showed inhibitory activity in vivo, and this work demonstrated the utility of peptoids for the development of intracellular PPI inhibitors. However, this peptoid-based approach requires the introduction of specific types of bulky N-substituents to achieve a constrained conformation. As a result, the choice of functional groups that can be introduced as N-substituents in ligand design is restricted.

Recently, we demonstrated that peptoids can be conformationally constrained by backbone modifications without introducing bulky N-substituents and macrocyclization. More specifically, the introduction of methyl groups on the backbone α-carbons of oligo-NSG was shown to conformationally constrain the peptoid, and the resulting oligo(N-substituted alanine) (oligo-NSA, Fig. 1a) stably formed an extended shape. The methyl groups on the α-carbons introduce steric repulsions on the backbone, known as pseudo-1,3-allylic strains, with the carbonyl oxygen in the preceding residue and the N-substituent of the following residue, and the bond rotation about the $\varphi$ and $\psi$ angles is restricted (Fig. 1b). As a result, the oligo-NSA structure is controlled on a per-residue basis by steric effects on the backbone and independently from N-substituents. Therefore, oligo-NSA can serve as a peptoid-based scaffold for PPI inhibitors with little restriction on the choice of functional groups introduced as
N-substituents.

Here, we propose oligo-NSA as a peptoid-based modularly reprogrammable template for the development of intracellular PPI inhibitors. Because the oligo-NSA structure is preorganized on a per-monomer basis irrespective of the structures of N-substituents, each N-substituent is modularly replaceable to optimize the binding affinity and membrane permeability, while preserving the backbone structure (Fig. 1c). More specifically, the monomers displaying N-substituents toward the target protein can be optimized to improve the affinity (highlighted in cyan in Fig. 1d right), while the monomers displaying N-substituents not facing the target protein can be optimized to improve membrane permeability without disturbing the interaction with the target protein (highlighted in green in Fig. 1d right). In this study, we demonstrated the utility of oligo-NSA as a modularly reprogrammable template for the development of intracellular PPI inhibitors by generating an inhibitor targeting the interaction between cancer-related MDM2 and p53.
Fig. 1  Design strategy for development of inhibitors of intracellular protein–protein interactions (PPIs) using oligo-NSA. (a) Chemical structures of oligo-NSG and oligo-NSA. (b) Schematic illustration of the rotational restrictions on the backbone about the dihedral angles φ and ψ in oligo-NSA exerted by pseudo-1,3-allylic strains. (c) The oligo-NSA structure is preorganized on a per-monomer basis. Oligo-NSAs with different functional groups as N-substituents with preserved backbone structures can be prepared by modularly replacing each monomer. (d) Design strategy for the development of cell-permeable PPI inhibitors. The rigid structure of oligo-NSA enables independent optimization of N-substituents to improve the binding affinity and membrane permeability, while preserving an extended backbone structure.
Results and discussion

Initial Design of Inhibitors of the Interaction between MDM2 and p53.

We recently reported the oligo-NSA 1-Pip as a ligand of MDM2 (Fig. 2a). In the previous study, 1-Pip was designed by mimicking the alignment of hot-spot residues in p53, the natural ligand of MDM2. p53 recognizes MDM2 with three hot-spot residues, Phe19, Trp23, and Leu26, displayed on the p53 transactivation domain (p53-TAD) (Fig. 2b). A model structure of oligo-NSA generated from quantum mechanical (QM) calculations (Fig. 2c) was superimposed on the crystal structure of p53-TAD bound to MDM2 (Fig. 2d). A good geometrical match between the N and Nα atoms of the 1st, 3rd, and 5th residues in the model oligo-NSA and the Cα and Cβ atoms of the three hot-spot residues in p53-TAD (RMSD = 0.88 Å) suggested that oligo-NSA can display substituents in the same orientations as the hot spot residues of p53. On the basis of this result, we designed the oligo-NSA 1-Pip, bearing hot spots of p53, and the oligo-NSA exhibited binding ability to MDM2.

However, the ligand designed in the previous study did not inhibit MDM2-p53 interaction in cellulo. The inhibitory activity of 1-Pip against intracellular MDM2-p53 interaction was examined in MDM2-overexpressing SJSA-1 cells. In MDM2-overexpressing cells, the ubiquitination and successive degradation of p53 or masking of the p53 transactivation domain by MDM2 negatively regulate p53 functions. If 1-Pip would inhibit the intracellular PPI, p53 degradation would be suppressed and the intracellular levels of p53 and p21, a transcriptional target of p53, would be increased (Fig. 2e). However, no detectable p53 or p21 were observed after treatment of SJSA-1 cells with 1-Pip even at 20 µM (Fig. 2f and S1). We initially assumed that the C-terminal cationic secondary amine adversely affected membrane permeability. Thus, we synthesized an oligo-NSA with the same sequence, but without the C-terminal piperazine (1, Fig. 2a), and evaluated its inhibitory activity. 1 showed in vitro inhibitory activity comparable to that of 1-Pip in a competitive fluorescence polarization (FP) assay using a previously reported MDM2 ligand, PMI peptide, as a fluorescent probe (Fig. 2g and Table S1). However, 1 also did not inhibit MDM2-p53 interaction in cellulo (Fig. 2f). The poor inhibitory activity of these initially designed inhibitors in cultured cells was considered to be due to their low affinity for MDM2 and their low membrane permeability.
Fig. 2  Initial design of oligo-NSA-based inhibitors of MDM2-p53 interaction. (a) Chemical structures of 1-Pip and 1. (b) Crystal structure of p53-TAD (cyan) bound to MDM2 (beige) (PDB: 1YCR). (c) Chemical structure (top) and model structure (bottom) of acetyl-N-ethylalanine pentamer generated by quantum mechanical calculations. (d) Overlay of p53-TAD (cyan) in 1YCR and model structure of the NSA pentamer (gray). The RMSD value for Cα and Cβ of Phe19, Trp23, and Leu26 in p53-TAD and N and Nα of 1st, 3rd, and 5th residues of oligo-NSA is indicated below the image. (e) Schematic illustration of the negative regulation of p53 function by MDM2 and activation of the p53 pathway via the inhibition of MDM2-p53 interaction. (f) Intracellular protein levels of p53, p21, and β-actin. SJSA-1 cells were treated with 20 µM oligo-NSA for 8 h and cell lysates were analyzed by western blotting. Lysate of SJSA-1 cells treated with a known inhibitor of the MDM2-p53 interaction, Nutlin-3a, was analyzed as a positive control. Representative results of three independent experiments are shown. Other results are shown in Fig. S1. (g) Inhibitory curves of 1-Pip and 1 against the interaction between
fluorescently labeled PMI peptide and MDM2 from a competitive FP assay. Y-axis indicates change in fluorescence anisotropy (ΔFA). Error bars represent standard deviations of triplicates.
**Design Strategy for Improved Binding Affinity and Membrane Permeability.**

The high modularity of oligo-NSAs was expected to allow independently optimizing each N-substituent to improve the binding affinity or membrane permeability, while preserving the backbone structure. We assumed that the binding affinity for MDM2 can be improved by optimizing the structures of the 1st, 3rd, and 5th N-substituents that face MDM2, while the membrane permeability can be improved by introducing lipophilic structures on the 2nd and 4th N-substituents that do not face MDM2. To support this assumption, we performed molecular dynamics (MD) simulations. First, MD simulations of 1 alone were performed using a CHARMM36m/CGenFF force field. From the obtained frames, the structure with the best geometrical match of N and N$_\alpha$ atoms of every other residue with the C$_\alpha$ and C$_\beta$ atoms of the three hot-spot residues in p53-TAD was selected. The structure was placed on the predicted binding site of MDM2, and the complex structure was subjected to MD simulations. A representative structure of the complex during the simulations is shown in Fig. 3a. In the complex, the 1st, 3rd, and 5th N-substituents on 1 were buried in the cleft of MDM2, and the 2nd and 4th N-substituents did not face MDM2. To analyze the engagement of each substituent in protein binding more quantitatively, the solvent accessible surface area (SASA) of each N-substituent during the simulations was calculated (Fig. S2). While the ratio of SASA to the whole surface area of the 1st, 3rd, and 5th N-substituents was below 0.1 on average, the ratios of SASA of the 2nd and 4th N-substituents remained high (0.7–0.9 on average). Thus, throughout the simulation time, the 1st, 3rd, and 5th N-substituents remained associated with the MDM2 surface, and the 2nd and 4th N-substituents were not facing MDM2, which supported our assumption.
Strategy for structural optimization to improve binding affinity and membrane permeability using oligo-NSA as a modularly reprogrammable template. (a) Molecular dynamics (MD) simulations of oligo-NSA 1 in complex with MDM2. A representative structure of the complex during the simulations is shown. (b) Submonomer synthesis of oligo-NSA. Each N-substituent is modularly introduced using aldehydes or alcohols as submonomers.
Structural Optimization to Improve Binding Affinity.

We first derivatized the 1st, 3rd, and 5th N-substituents to improve the binding affinity of the oligo-NSA 1. N-substituents were introduced by previously reported reductive amination\textsuperscript{28,33} or Fukuyama-Mitsunobu reaction\textsuperscript{34} using commercially available aldehydes or alcohols as submonomers (Fig. 3b). This submonomer synthetic method allows the modular replacement of each N-substituent. We independently optimized the 1st, 3rd, and 5th substituents, and several of the tested modifications showed improved inhibitory activity against the interaction between MDM2 and PMI peptide in a competitive FP assay (Fig. 4a, S3, and S4 and Table S2). Modifications of the R\textsubscript{1} substituent did not improve affinity (compounds S1–S3). Introduction of chloride to the indole group at R\textsubscript{3}, especially, substitution to a 6-chloroindolylmethyl group, improved the binding affinity by 2–11-fold (compounds 2 and S4). Compound S5, in which the indolylmethyl group at R\textsubscript{3} in 1 is replaced with a 4-chlorophenethyl group, also exhibited improved binding affinity. At the R\textsubscript{5} position, the neohexyl group (compound 3) was preferred over the isopentyl group, n-butyl group (compound S6), and cyclohexylmethyl group (compound S7). Based on these results, we synthesized and evaluated the binding affinity of compound 4 bearing benzyl, 6-chloroindolylmethyl, and neohexyl as the 1st, 3rd, and 5th N-substituent, respectively. The $K_i$ value of 4 was 0.34 µM, which is 15-fold stronger than that of the original ligand 1. These results demonstrated that the rigid backbone of oligo-NSA enables independent optimization of each substituent to improve the binding affinity.
Fig. 4 Structural optimization of oligo-NSA to improve binding affinity and membrane permeability. (a) Chemical structures of substituents at R$_1$, R$_3$, and R$_5$ and $K_i$ values of compounds 1–4. $K_i$ values were determined by a competitive FP assay using fluorescently labeled PMI peptide and MDM2. (b) Chemical structures of substituents at R$_2$ and R$_4$, AlogP values, $P_e$ values, and $K_i$ values of compounds 4–9. AlogP values are indices of lipophilicity of compounds. $P_e$ values were determined by a Caco-2 assay. $K_i$ values were determined by a competitive FP assay using fluorescently labeled PMI peptide and MDM2. n.d.: Not determined due to low water solubility in aqueous solution. *p < 0.05 compared with compound 4.
**Structural Optimization to Improve Membrane Permeability.**

We next designed derivatives of 4 to improve membrane permeability by modifying the substituents R₂ and R₄. First, the membrane permeability of 4 was measured by a Caco-2 assay (Fig. 4b). 4 showed only modest membrane permeability, with an effective permeability constant ($P_e$) of $0.58 \pm 0.20 \times 10^{-6}$ cm/s. To adjust the lipophilicity to a range preferable for membrane permeability, we synthesized compounds 5–9 bearing substituents with different lipophilicity, ethyl (Et), $n$-propyl (n-Pr), $n$-butyl (n-Bu), 2-methoxyethyl (Moe), or 2-hydroxyethyl (Hoe) groups, at the R₂ and R₄ positions (Fig. 4b), and evaluated their membrane permeability. Differences in the $P_e$ value were observed in the Caco-2 assay, and introduction of the Et groups (compound 5) enhanced the $P_e$ value by 2.4-fold. 6 and 7, with more lipophilic substituents, were too hydrophobic and thus, not soluble in aqueous buffer, and 8 and 9, with more hydrophilic substituents, showed lower $P_e$ values. These results are consistent with the fact that moderate lipophilicity is important for high membrane permeability and too high lipophilicity results in low water solubility.³⁵,³⁶ We also evaluated the permeability of 4–9 by a parallel artificial membrane permeability assay (PAMPA) (Table S3). $P_e$ values showed the same tendencies as in the Caco-2 assay, and oligo-NSA 5, bearing Et groups, showed the highest membrane permeability. These results demonstrated that the membrane permeability of oligo-NSA can be improved by appropriately modulating the lipophilicity via modification of the N-substituents.

We confirmed that the derivatization of R₂ and R₄ did not interrupt the interactions of 4, 5, 8, and 9 with MDM2 in a competitive FP assay (Fig. 4b and S5).

The results of the competitive binding assay and the permeability assay suggested that the membrane permeability of oligo-NSA can be improved without adversely affecting the binding ability by introducing substituents with appropriate lipophilicity at R₂ and R₄ that are not facing the interaction surface.
Evaluation of Backbone Preservation upon Modification of the N-Substituents.

To validate the assumption that the N-substituents of oligo-NSA can be replaced without disturbing the extended shape of the backbone, we conducted structural analysis.

The structural similarity of 1–5, 8 and 9 was first evaluated by circular dichroism (CD) measurements (Fig. S6). All oligo-NSAs exhibited similar spectral shapes; all spectra had a maximum around 195 nm and a minimum around 225 nm. These spectral shapes also resemble reported spectra of oligo-NSAs forming β-strand structures,\(^{28}\) which suggested that all the oligo-NSAs maintained the extended backbone shape.

To obtain more detailed structural information, we conducted NMR studies of the original oligo-NSA, 1, and the most potent oligo-NSA, 5. To facilitate obtaining the compounds at the multi-milligram scale, which is required for NMR analysis, these oligo-NSAs were synthesized with C-terminal piperazine (1-Pip and 5-Pip), which gives higher yields than the oligo-NSA 1 and 5 with C-terminal amide. Spatially proximal protons on the backbones of 1-Pip and 5-Pip were determined by ROESY measurements (Fig. S7 and S8). The α-protons, β-protons, and Nα protons of the compounds on the \(^1\)H NMR spectra were assigned with the aid of COSY, \(^{13}\)C NMR, HMQC, and HMBC spectra (Fig. S9–S18). For both compounds, NOE signals indicating rotational restrictions about ϕ and ψ via pseudo-1,3-allylic strains were observed (Fig. 5a–c). These results supported the preservation of the extended backbone structure before and after modification of the N-substituents.

To assess the dynamics of each oligomer structure, we conducted MD simulations. First, simulations of oligo-NSA 5 alone were conducted, and the results were evaluated together with the MD simulations of oligo-NSA 1 performed to prepare the model complex structure of 1 and MDM2 shown in Fig. 3a. Representative structures of 1 and 5 during MD simulations were generated by clustering the trajectories (Fig. S19), and the representative structures were superimposed over each other (Fig. 5d and e). The overall backbone structures were maintained in the extended shape throughout the simulation period, although some degree of structural fluctuation was observed for both oligomers (Fig. S20). The maintenance of the backbone structures was also suggested from distributions of the backbone dihedral angles, \(\varphi\) and \(\psi\) (Fig. S21). The \(\varphi\) and \(\psi\) angles were restricted around the region with low energy on the previously reported energy diagram during the majority of the simulation time.

Next, we conducted MD simulations of 5 in complex with MDM2, and the results were compared with the MD simulations of 1 in complex with MDM2 shown in Fig. 3a to assess the effect of the modification on the binding mode (Fig. 5f and g and S22–S24). Throughout the simulations, the N-substituents of the 1st, 3rd, and 5th residues of both oligomers were nearly completely buried in the p53-binding cleft of MDM2, while the N-substituents of the 2nd and 4th residues were not associated
with MDM2 and exposed to the solvent as shown by the SASA plot (Fig. S25).

These results supported the validity of our design principle, in which each N-substituent is independently optimized to improve the binding affinity and membrane permeability.
Fig. 5  Structural analysis of MDM2-binding oligo-NSAs. (a) Schematic illustration of the preferred conformations via pseudo-1,3-allylic strains. Red and blue arrows indicate spatially proximal protons via rotational restrictions of the backbone dihedral angles. Part of the three-dimensional model structure is shown next to each Newman diagram to visualize the proximities of the protons. (b, c) Summary of spatially proximal protons in 1-Pip (b) and 5-Pip (c) obtained from ROESY measurements. Protons with NOEs that suggest rotational restrictions about \( \phi \) (red arrows) and \( \psi \) (blue arrows) are indicated. (d, e) Overlay of five representative structures of 1 (d) and 5 (e) during MD simulations. Dominant conformations were determined by clustering analysis, and the backbones of the representative structures from each of the top five clusters were overlaid. (f, g) Representative structure of 1 (shown in cyan in f) and 5 (shown in pink in g) in complex with MDM2 during the MD simulations. The representative structures were determined by clustering analysis.
Evaluation of Intracellular Activities.
Finally, we evaluated the intracellular activity of the optimized oligo-NSA with improved binding affinity and membrane permeability. Intracellular levels of p53 and p21 in SJSA-1 cells with and without oligo-NSA 1 and 5 were analyzed by western blotting. Treatment of SJSA-1 cells with 5 for 8 h led to a dose-dependent increase in the levels of p53 and p21, whereas treatment with 1, the initial oligo-NSA before optimization of the N-substituents, showed no effects on the p53 and p21 levels (Fig. 6a, S26 and S27). This result indicated that optimization of the N-substituents led to improved intracellular activity. Furthermore, we evaluated the activity of 5-Rv, which is the reverse sequence of 5. 5-Rv showed a substantially lower inhibitory activity than 5 in vitro (Fig. S28) and had no effects on intracellular p53 and p21 levels (Fig. 6a, S26 and S27). This result suggested that p53 pathway activation by 5 is due to selective interaction of 5 with MDM2.

Next, we evaluated the apoptosis-inducing ability of 5 by staining SJSA-1 cells treated with 5 with Annexin V-FITC and propidium iodide (PI) (Fig. 6b and S29–S31). Treatment with 5 increased the ratios of Annexin V-positive cells and Annexin V- and PI-double positive cells, which indicates the induction of apoptosis. The degree of apoptosis induction increased in a dose-dependent manner (Fig. 6c). Consistent with the western blotting results, little apoptosis induction was observed in cells treated with 5-Rv. This result suggested that cell apoptosis induced by 5 is due to its interaction with MDM2, and not due to non-specific toxicity of the oligo-NSA. Furthermore, treatment with 5 had little effect on SW480 cells, which are mutant p53-bearing cancer cells (Fig. 6c and S32–S34), although high basal p53 levels were observed irrespective of the compounds used for treatments (Fig. S35 and S36). Thus, cell apoptosis induced by 5 was suggested to depend on p53 function. Therefore, 5 was suggested to trigger p53 pathway-dependent apoptosis, further supporting the selective PPI inhibitory effect of the oligo-NSA.

Altogether, we successfully developed a peptoid active in cells by using oligo-NSA as a modularly reprogrammable template and independently optimizing each N-substituent.
Fig. 6  Evaluation of inhibitory activity of oligo-NSAs in cellulo. (a) Intracellular protein levels of p53, p21, and β-actin in MDM2-overexpressing SJSA-1 cells treated with oligo-NSAs. SJSA-1 cells were incubated with 0, 2.5, 5, or 10 µM 5, 10 µM 1, 10 µM 5-Rv, or 10 µM Nutlin-3a for 8 h and cell lysates were analyzed by western blotting. Representative results of three independent experiments are shown. Other results are shown in Fig. S26. (b) Measurements of apoptotic cells by staining with Annexin V-FITC and propidium iodide (PI). After treatment with 10 µM 5 for 48 h, SJSA-1 cells were labeled with Annexin-V and PI and analyzed by flow cytometry. Representative results of three independent experiments are shown. Other results are shown in Fig. S29–S31. (c) Apoptotic responses of SJSA-1 cells (left) and mutant-p53 SW480 cells (right) to 1, 5, 5-Rv, and Nutlin-3a. Error bars represent standard deviations of three independent experiments.
Conclusions
In this study, we demonstrated that the rigid structure of oligo-NSA enables independent optimization of N-substituents for improving the binding ability and membrane permeability. Specific substituents are not required to achieve a constrained scaffold structure and thus, each substituent can be optimized without restriction on the choice of N-substituents. This modular approach using oligo-NSA as a reprogrammable template enabled facile development of an intracellular PPI inhibitor that induces cell apoptosis.

Peptoid-based approaches for developing PPI inhibitors have been faced with the challenge that the intrinsically flexible backbone of oligo-NSG often leads to low protein affinity. The introduction of bulky N-substituents is effective to constrain the oligo-NSG conformation, but it hampers structural optimization for affinity maturation due to the requirements of introducing the conformationally constraining elements on the N-substituents. The rigid backbone of oligo-NSA resolved the problem and liberated the N-substituents from structural ordering. Thus, rapid optimization of the N-substituents for high affinity and high membrane permeability was realized using oligo-NSA as a template structure.

Low-molecular weight compounds with constrained conformations, such as terphenyl, oligobenzamide, pyrrolopyrimidine, and oligooxopiperazine have been also reported to serve as template structures for PPI inhibitors, similar to oligo-NSA. Among these molecules, oligo-NSA exhibits the highest modularity; the oligomer is reprogrammable per N-substituted alanine module and the module can be easily constructed on resin using Fmoc-Ala-OH and an aldehyde or an alcohol. This high modularity is useful for rapidly optimizing both affinity and physicochemical properties, such as membrane permeability, of PPI inhibitors, as demonstrated in this study.

In conclusion, the peptoid-based modularly reprogrammable template will facilitate the development of inhibitors of diverse intracellular PPIs and expand the utility of peptoids in biomedical applications.

Author contributions
J.M. and S.S. conceived and directed the study. Y.F. contributed to the execution of this study. Y.F. synthesized compounds and conducted in vitro assays, cellular assays, and structural analysis. M.Y., D.K., and K.T. organized and/or conducted computational studies. Y.F., J.M., and S.S. wrote the manuscript and all authors approved the manuscript.

Conflicts of interest
The authors declare the following competing financial interest: J.M., Y.F., and S.S. have filed a patent application (PCT/JP2019/124016).

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Supplementary Information

Peptoid-Based Reprogrammable Template for Cell-Permeable Inhibitors of Protein–Protein Interactions

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Table of Contents

Materials and Methods ..........................................................................................4
  Abbreviations for chemical compounds...............................................................4
  General remarks for synthesis..............................................................................4
  General procedures for synthesis of oligo-NSAs with piperazinyl C-termini. ..........4
  General procedures for synthesis of oligo-NSAs with amide C-termini. ...............5
  Coupling reaction using COMU.........................................................................5
  Coupling reaction using EEDQ..........................................................................6
  Introduction of a substituent on resin-bound primary amine by reductive amination..........................6
  Introduction of an indolylmethyl group derivative on resin-bound primary amine by reductive amination. .................................................................6
  Acetylation of N-terminal amine.........................................................................7
  Removal of adducted acetyl group on indole......................................................7
  Synthesis of oligo-NSA 1–9.............................................................................8
  Synthesis of oligo-NSA S1–S7........................................................................12
  Synthesis of fluorescently labeled PMI peptide (Fluorescein-TSFAEYWNLLSP–NH2, Flu-PMI) for FP assay........................................................................15
  Recombinant expression and purification of MDM2.........................................15
  Fluorescence polarization (FP) binding assay of fluorescently labeled PMI peptide .................................................................15
  FP competitive binding assay of oligo-NSA derivatives....................................16
  Cell monolayer permeability assay....................................................................16
  Parallel artificial membrane permeability assay (PAMPA)................................17
  Calculation of ALogP values............................................................................17
  Cell culture.........................................................................................................17
  Evaluation of intracellular levels of p53 and p21 by western blotting...................18
  Evaluation of apoptosis-inducing ability by Annexin V assay.............................18
  CD studies.........................................................................................................19
  NMR spectroscopic studies...............................................................................19
  General remarks for computational studies......................................................19
  Molecular dynamics (MD) simulations..............................................................19
  Preparation of a template structure of acetyl-N-ethylalanine pentamer dimethylamide.................................21
Materials and Methods

Abbreviations for chemical compounds.
TEAA, Triethylammonium acetate; DMSO, Dimethylsulfoxide; DMF, N,N-Dimethylformamide; Fmoc, 9-Fluorenlymethoxycarbonyl; COMU, (1-Cyano-2-ethoxy-2-oxoethylideneaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate; EEDQ, 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; TFA, Trifluoroactic acid; DCM, Dichloromethane; DIPEA, N,N-Diisopropylethylamine; NMP, N-Methylpyrrolidone; TMOF, Trimethyl orthoformate; Ns, o-Nitrobenzenesulfonyl; DIAD, Diisopropyl azodicarboxylate; TMP, 2,4,6-Trimethylpyridine; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; DIC, N,N'-Diisopropylcarbodiimide; HOBt, 1-Hydroxybenzotriazole

General remarks for synthesis.
Chemicals and solvents used in this study were purchased from commercial suppliers and used without further purification. Preparative high performance liquid chromatography (HPLC) was performed on a Prominence HPLC system (Shimazu) with a 5C18-MS-II column (Nacalai tesque, 10 mm I.D.×150 mm, 34355-91). Analytical HPLC was performed on a Prominence HPLC system with a 5C18-AR-II column (Nacalai tesque, 4.6 mm I.D.×150 mm, 38144-31). HRMS data was obtained using micrOTOF II (Bruker Daltonics).

General procedures for synthesis of oligo-NSAs with piperazinyl C-termini.
Trityl chloride resin (#A00220, Watanabe Chemical Industries) was used for synthesis. Objective oligomers were synthesized on resin and cleaved as previously reported. Before cleaving the oligomers from resin, acetyl groups adducted to indole were removed by reduction using NaBH₄. Detailed procedures about each reaction step are described below. The crude product was dissolved in acetonitrile and water, and purified by a reversed phase column on HPLC using acetonitrile and 100 mM TEAA (pH 7.0) as solvents. After lyophilization, obtained compounds were dissolved in DMSO and quantified from UV absorbance derived from indole (ε = 5360 cm⁻¹·M⁻¹ at 280 nm determined from 3-hydroxymethyindole) or 6-chloroindole (ε = 4230 cm⁻¹·M⁻¹ at 286 nm determined from 6-chloro-3-hydroxymethyindole). Yields were determined by comparing with the amount of the first Fmoc-Ala-OH loaded on resin quantified as previously reported. The purified products were analyzed on a reversed phase column by HPLC and ESI-TOF MS.
**General procedures for synthesis of oligo-NSAs with amide C-termini.**

Fmoc-protected Sieber amide resin (#A00258, Watanabe Chemical Industries) was used for synthesis. The resin was swelled with DMF for 30 min and then Fmoc protecting group was removed by treatment with 20% piperidine/DMF (3 min and 12 min). Coupling reaction of the first Fmoc-Ala-OH was conducted using COMU as a coupling reagent. After deprotection of Fmoc group, an N-substituent was introduced by reductive amination. Objective oligomers were synthesized on resin as previously reported\(^1\) by repeating the cycle of coupling of Fmoc-Ala-OH using EEDQ as a coupling reagent, deprotection of Fmoc, and introduction of an N-substituent by reductive amination. For 2-methoxyethyl group, 2-hydroxyethyl group, and 4-chlorophenylethyl group, substituents were introduced on resin using alcohols by Fukuyama-Mitsunobu reaction.\(^2\) For residues with N-methyl substituents, coupling reaction of Fmoc-N-Me-Ala-OH was conducted instead of introducing an N-substituent by reductive alkylation or Fukuyama-Mitsunobu reaction. Before cleaving oligomers from resin, acetyl groups adducted to indole were removed by reduction using NaBH\(_4\). Detailed procedures about each reaction step are described below. The oligomers were cleaved by treatment of the resin with 1% TFA/DCM for 10 s. The solution was transferred to a glass vial containing pyridine/methanol to quench TFA. This cleavage process was repeated five times. The resin was washed with methanol three times and the methanol solutions were collected in the glass vial. The solvents were removed under reduced pressure. The crude product was dissolved in acetonitrile and water, and purified by a reversed phase column on HPLC using acetonitrile and 100 mM TEAA (pH 7.0) as solvents. After lyophilization, obtained compounds were dissolved in DMSO and quantified from UV absorbance derived from indole (\(\varepsilon = 5360 \text{ cm}\(^{-1}\)·M\(^{-1}\) at 280 nm determined from 3-hydroxymethyindole), 6-chloroindole (\(\varepsilon = 4230 \text{ cm}\(^{-1}\)·M\(^{-1}\) at 286 nm determined from 6-chloro-3-hydroxymethyindole), or 5-chloroindole (\(\varepsilon = 4300 \text{ cm}\(^{-1}\)·M\(^{-1}\) at 288 nm determined from 5-chloro-3-hydroxymethyindole). Yields were determined by comparing with the loading amount of the resin. The purified products were analyzed on a reversed phase column by HPLC and ESI-TOF MS.

**Coupling reaction using COMU.**

![Coupling reaction using COMU](image)

Resin-bound primary amine was coupled with Fmoc-Ala-OH as follows. First, resin was washed with DMF three times. Then, a DMF solution of Fmoc-Ala-OH (4 equiv., 0.2 M), COMU (4 equiv., 0.2 M) and
DIPEA (8 equiv., 0.4 M) was added to the resin and the reaction vessel was shaken for 1 h. After the solution was removed, the resin was washed with DMF three times.

**Coupling reaction using EEDQ.**

Resin-bound secondary amine was coupled with Fmoc-Ala-OH as follows. First, resin was washed with dioxane three times. Fmoc-Ala-OH (4 equiv.) and EEDQ (4 equiv.) were dissolved in dioxane to prepare 0.2 M solution and the mixture was shaken. After 30 min, the solution was added to the resin and the reaction vessel was shaken for 3 h at 60ºC. This coupling reaction was repeated once more when the N-terminal substituent on the resin is larger than methyl. After the reaction, the resin was washed with dioxane and DMF three times each.

**Introduction of a substituent on resin-bound primary amine by reductive amination.**

Resin was washed with DMF three times, then incubated with 1 M (20 equiv.) of an aldehyde solution in DMF for 1 h. The solution was filtered off and the resin was quickly washed with DMF and DCM twice each. A 3/1 DCM/methanol solution (20 µL/µmol of loading amount of resin) and 10 equiv. of NaBH₄ were added to the resin and shaken for 30 min with the reaction vessel standing and open to the atmosphere. The resin was washed with methanol five times then with DCM and DMF three times each.

**Introduction of an indolylmethyl group derivative on resin-bound primary amine by reductive amination.**

Resin was washed with TMOF three times, then incubated with a suspension of aldehyde (10 equiv.) in TMOF (20 µL/µmol of loading amount of resin) with 1% acetic acid as an additive for 1 h. 10 equiv. of NaBH(OAc)₃ was added to the reaction mixture and shaken for additional 30 min. The resin was washed
with methanol five times then with DMF three times.

**Introduction of an N-substituent on resin-bound primary amine by Fukuyama- Mitsunobu reaction.**

Resin was washed with NMP three times and then incubated with a solution of Ns-Cl (4 equiv., 0.1 M) and TMP (10 equiv., 0.25 M) in NMP for 15 min. The solution was filtered off and the resin was washed with NMP and toluene three times each. A solution of alcohol (8 equiv., 0.8 M) and triphenylphosphine (4 equiv., 0.4 M) in toluene was added to the resin, and a solution of DIAD (4 equiv., 0.4 M) in toluene was added dropwise to give a final concentration of 0.4 M of alcohol, 0.2 M of triphenylphosphine and 0.2 M of DIAD. The reaction vessel was capped and allowed to shake for 6 h at 60°C. After the resin was washed with toluene and DMF three times each, \( \text{o-NBS} \) groups were removed by the treatment with a solution of \( \beta \)-mercaptoethanol (8 equiv., 0.4 M) and DBU (4 equiv., 0.2 M) in NMP for 10 min. This deprotection process was repeated twice more and the resin was washed with DMF three times.

**Acetylation of N-terminal amine.**

Resin was washed with DMF three times. A DMF solution of acetic anhydride (10 equiv., 0.5 M) and DIPEA (20 equiv., 1 M) was added to the resin and the reaction vessel was shaken for 1.5 h. This procedure was repeated once more, and the resin was washed with DMF three times.

**Removal of adducted acetyl group on indole.**

Resin was washed with DCM three times. A solution of 3/1 DCM/methanol (20 µL/µmol of loading
amount of resin) and 10 equiv. of NaBH₄ was added to the resin and shaken for 30 min with the reaction vessel standing and open to the atmosphere. The resin was washed with methanol five times then with DCM three times.

**Synthesis of oligo-NSA 1–9.**

**Synthesis of oligo-NSA 1.**

41 mg of Sieber amide resin (0.48 mmol/g, 20 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, indole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 6%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₀H₅₇N₇NaO₆⁺ 754.4263; Found 754.4254.

**Synthesis of oligo-NSA 1-Pip.**

40 mg of trityl chloride resin (1.96 mmol/g, 78 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, indole-3-carboxaldehyde and benzaldehyde were used as submonomers. After loading first Fmoc-alanine, the loading amount was determined as 1.2 mmol/g. Yield: 56%. HRMS (ESI-TOF MS) m/z: [M + H]⁺ Calcd for C₄₄H₆₅N₆O₆⁺ 801.5022; Found 801.5029.
Synthesis of oligo-NSA 2.

40 mg of Sieber amide resin (0.48 mmol/g, 19 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 7%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₀H₅₆ClN₇NaO₆+ 788.3873; Found 788.3893.

Synthesis of oligo-NSA 3.

41 mg of Sieber amide resin (0.48 mmol/g, 20 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, 3,3-dimethylbutyaldehyde, indole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 6%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₁H₅₉N₇NaO₆+ 768.4419; Found 768.4436.

Synthesis of oligo-NSA 4.

83 mg of Sieber amide resin (0.48 mmol/g, 40 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, 3,3-dimethylbutyaldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 8%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₁H₅₈ClN₇NaO₆+ 802.4029; Found 802.4027.
Synthesis of oligo-NSA 5.

100 mg of Sieber amide resin (0.48 mmol/g, 48 μmol) was used for synthesis. Fmoc-Ala-OH, acetaldehyde, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 2%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₃H₆₂ClN₇NaO₆⁺ 830.4342; Found 830.4331.

Synthesis of oligo-NSA 5-Pip.

80 mg of trityl chloride resin (1.96 mmol/g, 156 μmol) was used for synthesis. Fmoc-Ala-OH, acetaldehyde, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. After loading first Fmoc-alanine, the loading amount was determined as 1.2 mmol/g. Yield: 41%. HRMS (ESI-TOF MS) m/z: [M + H]⁺ Calcd for C₄₇H₇₀ClN₈O₆⁺ 877.5101; Found 877.5094.

Synthesis of oligo-NSA 5-Rv.

99 mg of Sieber amide resin (0.48 mmol/g, 48 μmol) was used for synthesis. Fmoc-Ala-OH, acetaldehyde, benzaldehyde, 6-chloroindole-3-carboxaldehyde and 3,3-dimethylbutyraldehyde were used as submonomers. Yield: 2%. HRMS (ESI-TOF MS) m/z: [M + Na]⁺ Calcd for C₄₃H₆₂ClN₇NaO₆⁺ 830.4342; Found 830.4304.
Synthesis of oligo-NSA 6.

34 mg of Sieber amide resin (0.48 mmol/g, 16 μmol) was used for synthesis. Fmoc-Ala-OH, Propionaldehyde, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 3%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{45}H_{66}ClN_{7}NaO_{6}^+ 858.4655; Found 858.4633.

Synthesis of oligo-NSA 7.

34 mg of Sieber amide resin (0.48 mmol/g, 16 μmol) was used for synthesis. Fmoc-Ala-OH, n-butyraldehyde, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 2%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{47}H_{70}ClN_{7}NaO_{6}^+ 886.4968; Found 886.4938.

Synthesis of oligo-NSA 8.

100 mg of Sieber amide resin (0.48 mmol/g, 48 μmol) was used for synthesis. Fmoc-Ala-OH, 2-methoxyethanol, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were
used as submonomers. Yield: 5%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Caled for C_{45}H_{66}Cl_{7}N_{7}NaO_{8}^+ 890.4554; Found 890.4511.

**Synthesis of oligo-NSA 9.**

![Chemical Structure]

100 mg of Sieber amide resin (0.48 mmol/g, 48 µmol) was used for synthesis. Fmoc-Ala-OH, 2-[(tert-butyldimethyl)silyl]oxy)ethanol, 3,3-dimethylbutyraldehyde, 6-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Before removal of acetyl group and cleavage from resin, tert-butyldimethylsilyl protecting groups were removed by treatment of the resin with 0.1 M tetrabutylammonium fluoride in 9/1 DMF/THF for 2 h. Yield: 1%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Caled for C_{43}H_{62}Cl_{7}N_{7}NaO_{8}^+ 862.4241; Found 862.4199.

**Synthesis of oligo-NSA S1–S7.**

**Synthesis of oligo-NSA S1.**

![Chemical Structure]

40 mg of Sieber amide resin (0.48 mmol/g, 19 µmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-\textit{N}-Me-Ala-OH, isovaleraldehyde, indole-3-carboxaldehyde and 4-chlorobenzaldehyde were used as submonomers. Yield: 4%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Caled for C_{40}H_{56}Cl_{7}N_{7}NaO_{6}^+ 788.3873; Found 788.3897.
Synthesis of oligo-NSA S2.

40 mg of Sieber amide resin (0.48 mmol/g, 19 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, indole-3-carboxaldehyde and 3-chlorobenzaldehyde were used as submonomers. Yield: 4%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{40}H_{56}ClN_{7}NaO_{6}^+ 788.3873; Found 788.3887.

Synthesis of oligo-NSA S3.

40 mg of Sieber amide resin (0.48 mmol/g, 19 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, indole-3-carboxaldehyde and 4-bromobenzaldehyde were used as submonomers. Yield: 2%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{40}H_{56}BrN_{7}NaO_{6}^+ 832.3368; Found 832.3376.

Synthesis of oligo-NSA S4.

40 mg of Sieber amide resin (0.48 mmol/g, 19 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, 5-chloroindole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 16%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{40}H_{56}ClN_{7}NaO_{6}^+ 788.3873; Found 788.3895.
Synthesis of oligo-NSA S5.

40 mg of Sieber amide resin (0.48 mmol/g, 19 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, isovaleraldehyde, 2-(4-chlorophenyl)ethanol and benzaldehyde were used as submonomers. Yield: 48%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{39}H_{57}ClN_6O_6^+ 763.3920; Found 763.3951.

Synthesis of oligo-NSA S6.

41 mg of Sieber amide resin (0.48 mmol/g, 20 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, n-butyraldehyde, indole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 6%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{39}H_{55}N_7NaO_6^+ 740.4106; Found 740.4122.

Synthesis of oligo-NSA S7.

41 mg of Sieber amide resin (0.48 mmol/g, 20 μmol) was used for synthesis. Fmoc-Ala-OH, Fmoc-N-Me-Ala-OH, cyclohexanecarboxaldehyde, indole-3-carboxaldehyde and benzaldehyde were used as submonomers. Yield: 7%. HRMS (ESI-TOF MS) m/z: [M + Na]^+ Calcd for C_{42}H_{39}N_7NaO_6^+ 780.4419; Found 780.4431.
Synthesis of fluorescently labeled PMI peptide (Fluorescein-TSFAEYWNLLSP–NH₂, Flu-PMI) for FP assay.

PMI peptide³ was synthesized by standard Fmoc solid phase peptide synthetic method on a fully automated parallel peptide synthesizer Syro I (Biotage) using Rink Amide resin (#A00172, Watanabe Chemical Industries) and Fmoc-amino acids. 50 mg of Rink Amide resin (0.48 mol/mg, 24 µmol) was used for synthesis. Coupling reaction was performed with DMF solution of Fmoc-amino acid (4 equiv., 0.2M), COMU (4 equiv., 0.2 M) and DIPEA (8 equiv., 0.4 M). Coupling reaction of 5(6)-carboxyfluorescein was performed manually. DMF solution of 5(6)-carboxyfluorescein (2.5 equiv., 0.1 M), DIC (2.5 equiv., 0.1 M) and HOBt (2.5 equiv., 0.1 M) was added to the resin with continuous shaking overnight. After removing the solution, the resin was washed with DMF three times and incubated with 20% piperidine/DMF for 45 min to remove any side products bearing additional carboxyfluoresceins. After removing the solution, the resin was washed with DMF, methanol and DCM three times each. Peptide was cleaved and deprotected by treating the resin with 95/2.5/2.5 TFA/TIPS/H₂O for 2 h. The solution was transferred to a recovery flask and TFA solution was removed under reduced pressure. Peptide was precipitated by adding diethylether to the flask. The precipitated crude product was dissolved in acetonitrile and water, and purified by a reversed phase column on HPLC using acetonitrile and water containing 0.1% TFA as solvents. The purified product was analyzed on a reversed phase column by HPLC and ESI-TOF MS. HRMS (ESI-TOF MS) m/z: [M + Na]+ Calcd for C₅₉H₁₀₅N₁₅NaO₂₅ + 1806.7298; Found 1806.7291.

Recombinant expression and purification of MDM2.

Human MDM2, corresponding to residues 17-125, was expressed using pGEX-6P-2 vectors in BL21 (DE3) cells grown in LB medium as previously described.¹ The pGEX-6P-2-MDM2 (17-125) was a gift from Gary Daughdrill (#62063, Addgene plasmid).⁴ After the removal of GST tag, the buffer was exchanged to 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and MDM2 was purified by size exclusion chromatography. Size exclusion chromatography was performed on an NGC chromatography system (Bio-Rad) with a HiLoad 16/600 Superdex 75 pg column (#28-9893-33, Cytiva). Fractions containing MDM2 were combined and the buffer was exchanged to PBS.

Fluorescence polarization (FP) binding assay of fluorescently labeled PMI peptide.

Serially diluted MDM2 solutions were mixed with fluorescently labeled PMI peptide (Flu-PMI) (10 nM) solutions in 384-well plate to prepare 15 µL of solutions in PBS (pH 7.4) containing 0.01% Tween 20. As a control, solutions of 10 nM Flu-PMI were also prepared. The plate was spun down at 500 × g for 5 min
and incubated at room temperature for 1 h. Fluorescence anisotropy (FA) was measured on a plate reader (TECAN, infinite M1000Pro) at 25°C. Excitation wavelength and emission wavelength were set to 470 nm and 521 nm, respectively. ΔFA values were determined from the average of three measurements of difference between solution with MDM2 and blank (without MDM2). Dissociation constants (KD) were calculated using parameters determined by fitting the plot to the Hill equation on ORIGIN (LightStone): y = y_{min} + (y_{max} - y_{min})/(1 + (K_D/x)^n). x, y, and n denote MDM2 concentration, ΔFA, and hill coefficient, respectively.

**FP competitive binding assay of oligo-NSA derivatives.**

Serially diluted oligo-NSA solutions were mixed with MDM2 (40 nM) and Flu-PMI solutions (10 nM) in 384-well plate to prepare 15 µL of solutions in PBS (pH 7.4) containing 0.01% Tween 20. As a control, solutions of 10 nM Flu-PMI without oligo-NSA and MDM2 were also prepared. FA was measured following the same procedures in the previous section. ΔFA values were determined from the average of three measurements of difference between solution with MDM2 and oligo-NSAs and blank (without MDM2 and oligo-NSA). IC_{50} values were determined by fitting the plot to the Hill equation on ORIGIN (LightStone): y = y_{max} - y_{max}/(1 + (IC_{50}/x)^n). x, y, and n denote inhibitor oligomer concentration, ΔFA and hill coefficient, respectively. Inhibition constants (Ki) were calculated using determined KD of the fluorescently labeled PMI peptide and IC_{50} as previously reported.\(^5\)

**Cell monolayer permeability assay.**

The assay was conducted using Millicell cell culture insert plates (Millipore). The filter area of the plate was 0.7 cm\(^2\), 0.42 × 10\(^5\) Caco-2 cells (#HTB-37, ATCC) in 400 µL of culture medium (DMEM with high glucose containing 10% Fetal bovine serum (FBS), 1% nonessential amino acids, and 1% penicillin-streptomycin solution) were spread to each insert chamber and 800 µL of the culture medium was added in each well of the receiver plate. The cell culture insert plates were incubated in a 5% CO\(_2\) incubator at 37°C for 22 days. During the incubation, medium was refreshed twice a week. After the incubation, the transepithelial electrical resistance in each well was measured to confirm that the value is > 300 W cm\(^2\), which suggest the formation of a cell monolayer. The medium both in apical and basolateral wells was removed using aspirator and an apical chamber and basolateral chamber was washed with 400 µL and 800 µL of transport buffer (HBSS buffer containing 10 mM HEPES (pH 7.4)), respectively. After the wash, an apical chamber and a basolateral chamber was filled with the same buffer solution and the plates were incubated at 37°C for 30 min. After removing solution in apical and basolateral wells, 420 µL of 10 µM oligo-NSA solution in transport buffer containing 1% DMSO was
added to each apical well and 20 µL of the solution was immediately recovered as sample at time zero. 800 µL of transport buffer containing 1% DMSO was added to each basolateral well and the plates were incubated at 37°C for 3 h. After the incubation, 20 µL and 200 µL of the solution in an apical well and a basolateral well, respectively, were recovered and analyzed by LC-MS/MS. The solution in apical and basolateral wells was removed using aspirator and 400 µL of 300 µM lucifer yellow solution in transport buffer containing 1% DMSO and 800 µL of transport buffer containing 1% DMSO were added to each apical chamber and basolateral chamber, respectively. An apical chamber and basolateral chamber was washed with 400 µL and 800 µL of transport buffer, respectively. After 1 h incubation in 5% CO₂ incubator at 37°C for 30 min, 200 µL of solution in basolateral chamber was transferred to a 96-well black plate and fluorescence from each well was measured by a plate reader with excitation wavelength of 485 nm and emission wavelength of 538 nm. Wells with 2 × 10⁻⁶ cm/sec for lucifer yellow was not recruited. Using the determined concentrations of oligo-NSAs in apical and basolateral wells, the permeability value (Pₑ) was calculated according to a previous report. Each compound was tested in triplicate or quadruple.

Parallel artificial membrane permeability assay (PAMPA).
The assay was conducted using Gentest Pre-coated PAMPA Plate System (Corning). The filter area of the plate was 0.3 cm². 300 µL of 10 µM solution of each oligo-NSA in 5% DMSO/100 mM phosphate buffer (pH7.4) was added to each donor well in the receiver microplate and 200 µL of 5% DMSO/PBS was added to each of the wells. For oligo-NSA 6 and 7, 0.1 and 1 µM solution, respectively, were used due to the low aqueous solubility of the compounds. The donor plate was docked on the acceptor well and the plates were incubated on a thermomixer (Eppendorf) for 4 h at 37°C. After the incubation, oligo-NSAs in acceptor and donor wells were quantitated by LC-MS/MS. Using the determined concentrations of oligo-NSAs in donor and acceptor wells, the permeability value (Pₑ) was calculated according to a previous report.

Calculation of ALogP values.
ALogP values were calculated using ALogP 2.1 from Virtual Computational Chemistry Lablatory.

Cell culture.
SJSA-1 and SW480 cells were cultured at 37°C in 5% CO₂ atmosphere in RPMI-1640 supplemented with 10% heat-inactivated FBS and 1% Antibiotic-Antimycotic (#09366-44, Nacalai Tesque).
Evaluation of intracellular levels of p53 and p21 by western blotting.
SJSA-1 or SW480 cells were seeded in 35-mm dishes (3 × 10^5 cells) and cultured in RPMI-1640 supplemented with 10% FBS and 1% Antibiotic-Antimycotic for 24 h. The medium was replaced, and the cells were treated with oligo-NSA derivatives or Nutlin-3a (#ab144428, Abcam) at an appropriate concentration in RPMI-1640 (10% FBS, 1% Antibiotic-Antimycotic, 0.1% DMSO) for 8 h at 37ºC in 5% CO₂ atmosphere. The cells were washed with PBS twice and lysed with lysis buffer (50 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 1 µg/mL leupeptin) supplemented with 1 mM AEBSF. The cell lysates were passed through 27 gauge needles followed by centrifugation at 12,000 × g for 30 min at 4 ºC, and then the supernatants were recovered. The total protein concentration of each cell lysate was determined by Bicinchoninic acid (BCA) assay, and then adjusted to the same value. The cell lysates containing 0.6 or 1 µg of total protein were subjected to SDS-PAGE and then transferred to the polyvinylidene difluoride (PVDF) membranes (#IPVH00010, Merck Millipore). The membranes were blocked with Blocking One (#03953-95, Nacalai Tesque) for 1 h. The membranes were incubated with anti-p53 (#DO-1, Santa Cruz), anti-p21 (1:200; #OP64, Merck), or anti-β-actin (1:3000; #4967, Cell Signaling Technology) antibodies at 4ºC overnight, followed by horseradish peroxidase conjugated anti-mouse immunoglobulins (1:3000; P0447, Dako) or anti-rabbit immunoglobulins (1:3000; P0488, Dako) secondary antibodies at room temperature for 1 h. The membranes were probed using ImmunoStar LD (#296-69901, Fujifilm Wako Pure Chemical Corporation), and chemiluminescence was detected using Ez-Capture MG AE9300H-CSP (ATTO).

Evaluation of apoptosis-inducing ability by Annexin V assay.
SJSA-1 or SW480 cells were seeded in 12-well tissue culture plates (1 × 10^5 cells per well), and cultured in RPMI-1640 supplemented with 10% FBS and 1% Antibiotic-Antimycotic for 24 h. The medium was replaced, and the cells were treated with oligo-NSA derivatives or Nutlin-3a at an appropriate concentration in RPMI-1640 (10% FBS, 1% Antibiotic-Antimycotic, 0.1% DMSO) for 48 h at 37ºC in 5% CO₂ atmosphere. Cells were washed with PBS twice. Culture medium and the first wash solution that may contain detached cells were collected. Cells attached to the plate were detached by incubation with TrypLE Express (#12605028, Thermo Fisher) at 37ºC for 5 min, and combined with collected cells in medium and PBS. Cells were collected by centrifugation at 500 × g for 5 min, and then washed with PBS twice. Apoptotic cells were detected by Annexin V-FITC apoptosis detection kit (#15342-54, Nacalai Tesque). Cells were suspended in the supplied binding buffer. Annexin V-FITC and propidium iodide (PI) solutions were added to the cell suspensions. The cell suspensions were incubated at room temperature for 15 min in the dark. The suspensions were diluted with the supplied binding buffer and analyzed by
flow cytometry (Guava easyCyte, Merck Millipore).

**CD studies.**

CD spectra of 50 µM oligo-NSAs in 20 mM phosphate buffer (pH 7.4) containing 5% methanol were measured at 25°C with a CD spectrometer (JASCO, J-1500) using 1 mm path length quartz cell (JASCO, 209J). Data pitch was set to 1 mm. Spectra were averaged from three scans. Spectral baseline was recorded using 20 mM phosphate buffer (pH 7.4) containing 5% methanol. All data points were baseline subtracted, converted to a uniform scale of molar ellipticity per residue and plotted.

**NMR spectroscopic studies.**

NMR spectra of oligo-NSA **1-Pip** and **5-Pip** were recorded at 15 mM in CDCl₃ on a JEOL ECS-400. ROESY, ¹H NMR, ¹³C NMR, COSY, HMQC, and HMBC spectrum of **1-Pip** were recorded with the following conditions. ROESY spectrum was recorded with relaxation delay of 1.5 s, mixing time of 0.25 s, and receiver gain of 42. COSY spectrum was recorded with relaxation delay of 1.5 s and receiver gain of 50. HMQC spectrum was recorded with x points of 2048, y points of 1024, relaxation delay of 1.5 s, and receiver gain of 50. HMBC spectrum was recorded with x points of 2048, y points of 1024, relaxation delay of 1.5 s, and receiver gain of 86.

ROESY, ¹H NMR, ¹³C NMR, COSY, HMQC, and HMBC spectrum of **5-Pip** were recorded with the following conditions. ROESY spectrum was recorded with relaxation delay of 1.5 s, mixing time of 0.25 s, and receiver gain of 44. COSY spectrum was recorded with relaxation delay of 1.5 s and receiver gain of 50. HMQC spectrum was recorded with x points of 2048, y points of 1024, relaxation delay of 1.5 s, and receiver gain of 50. HMBC spectrum was recorded with x points of 2048, y points of 1024, relaxation delay of 1.5 s, and receiver gain of 88.

Chemical shifts of ¹H NMR, ¹³C NMR, COSY, HMQC, HMBC, and ROESY spectrum are reported in ppm relative to internal standards (δH, tetramethylsilane 0 ppm; δC, tetramethylsilane 0 ppm). Assignment of ¹H NMR was assisted by COSY, HMQC, and HMBC spectrum.

**General remarks for computational studies.**

All QM calculations were carried out with the Gaussian16 package⁸ and all MD simulations were carried out with the Gromacs 2020.4 package⁹ and the CHARMM36m/CGenFF force field.¹⁰,¹¹

**Molecular dynamics (MD) simulations.**

For each simulation, a conformation derived from density functional theory (DFT) calculations was used
as the initial structure. More specifically, the DFT-optimized structure of acetyl-N-ethylalanine pentamer dimethylamide was used as a template and N-substituents of each compound were grafted on the template. When N-substituents were grafted, redundant structures were removed from the template. The substituent-grafted structures were minimized by molecular mechanics calculations using MMFF94s as a force field with fixed backbone atoms (N, Nα, Nβ, Cα, Cβ, and C=O) except for hydrogens. The energy minimization was conducted using Avogadro: an open-source molecular builder and visualization tool. Version 1.1.1. (http://avogadro.cc/). The procedure for preparation of the template structure of acetyl-N-ethylalanine pentamer dimethylamide was described in Supplementary Note. The initial conformation was solvated with TIP3P water in a rectangular box such that the minimum distance to the edge of the box was 15 Å under periodic boundary conditions. Na and Cl ions were added to imitate a salt solution of concentration 0.15 M. The system was energy-minimized by the steepest descent algorithm (5,000 steps), and heated from 50 K to 298 K during 200 ps, and the simulations were continued by 300 ps with NVT ensemble, where peptoid atoms were held fixed whereas non-peptoid atoms freely moved. Further simulations were performed with NPT ensemble at 298 K for 500 ns without any restraints other than the LINCS algorithm to constrain bonds involving hydrogen atoms. For each system, the simulation was repeated 5 times with different initial velocities (i.e. 2.5 µs in total for each peptoid). The time step was set to 2 fs throughout the simulations. A cutoff distance of 12 Å was used for Coulomb and van der Waals interactions. Long-range electrostatic interactions were evaluated by means of the particle mesh Ewald method. A snapshot was saved every 100 ps. For the analysis of each trajectory, we employed the last 400 ns. Representative structures were obtained from clustering of the trajectory. More specifically, clustering analysis was conducted based on the coordinates of five backbone α-carbons of oligo-NSAs using 1 frame per 20 frames from the total 20,005 frames of 5 simulations with an ensemble clustering tool implemented with UCSF Chimera. Representative structures of top 5 clusters were selected as dominant conformations.

MD simulations of the complexes of MDM2 and oligo-NSAs were performed in a similar manner with the above MD simulations of the oligo-NSA alone. The CHARMM36m force field was used to model the MDM2 in complex with oligo-NSAs. For each simulation, an initial structure of the complex was generated using a conformation of oligo-NSA from the MD simulations and a previously reported crystal structure of MDM2. More specifically, for oligo-NSA, a structure with orientations of N-substituents matching to the orientations of three hot-spot residues of p53 (Phe19, Trp23, Phe26) was selected from the trajectory of the above-described MD simulations of each oligo-NSA alone and used as the initial structure. For MDM2, a structure from a co-crystal structure of MDM2 and p53 (PDB 1YCR) was used as the initial structure. Missing residues in the MDM2 structure were complemented using Modeller.
The structure of p53 in the co-crystal structure of MDM2 and p53 (PDB 1YCR) were then replaced with the initial structures of oligo-NSA, and the resulting complex was used as the initial coordinates for the subsequent MD simulations. For the analysis of each trajectory, we employed the last 400 ns. Representative conformations were obtained from clustering of the trajectory. More specifically, clustering analysis was conducted based on the coordinates of five backbone $\alpha$-carbons of oligo-NSAs using 1 frame per 20 frames from the total 20,005 frames of 5 simulations with an ensemble clustering tool implemented with UCSF Chimera. Representative structures of top 5 clusters were selected as dominant binding mode. SASA of each N-substituent on oligo-NSAs in complex with MDM2 was calculated with Gromacs 2020.4 package. Calculated SASA was normalized by SASA calculated from trajectory where MDM2 was removed, and the normalized SASA during simulations were plotted and evaluated.

**Preparation of a template structure of acetyl-$N$-ethylalanine pentamer dimethylamide.**

The energy landscapes about $\phi$ and $\psi$ of acetyl-$N$-methylalanine dimethylamide were generated by combinatorially fixing $\phi$ and $\psi$ at every 15° from $-180^\circ$ to $180^\circ$. Each conformer was optimized at the B3LYP/6-31G* level using a Self-consistent reaction field (SCRF) model with water as the solvent. The $\omega$ angles were fixed to $180^\circ$ through the calculations. Angles with the lowest energy were determined to be $(\phi, \psi) = (-120^\circ, 90^\circ)$. Next, the energy landscapes about $\chi$ of acetyl-$N$-ethylalanine dimethylamide were generated by optimizing a conformer with $\chi$ angle of $-180^\circ$ to $180^\circ$ with 10° increment. The calculation started with an initial conformation of $(\chi, \phi, \psi) = (0^\circ, -120^\circ, 90^\circ)$. Angles with the lowest energy were determined to be $\chi = -90^\circ$ or $90^\circ$. Finally, a conformation of acetyl-$N$-ethylalanine pentamer dimethylamide with dihedral angles $(\chi, \phi, \psi, \omega)$ of $(-90^\circ, -120^\circ, 90^\circ, 180^\circ)$ were optimized at the B3LYP/6-31G* level using a SCRF model with water as the solvent.
Supporting Figures & Tables

**Fig. S1**  Intracellular protein levels of p53, p21, and β-actin analyzed by western blotting. SJSA-1 cells were treated with 20 µM 1-Pip or 1 or 10 µM Nutlin-3a for 8 h. (a, b) Results of two of three independent experiments are shown. Another result is shown in Fig. 2f. (c) Uncropped image of Fig. 2f. (d, e) Uncropped image of Fig. S1a and b.
Table S1  Parameters obtained from the curve fitting (Hill equation: $y = y_{\text{max}} - \frac{y_{\text{max}}}{(1 + (\text{IC}_{50}/x)^n)}$) of the data in a competitive FP assay of 1-Pip and 1. Inhibitory curves are shown in Fig. 2g.

| Compound | $y_{\text{max}}$ (µM) | IC$_{50}$ (µM) | n         |
|----------|-----------------------|---------------|-----------|
| 1-Pip    | 39.3 ± 0.3            | 12 ± 1        | 0.96 ± 0.05 |
| 1        | 41.2 ± 0.4            | 18 ± 1        | 1.06 ± 0.05 |
Fig. S2  Ratio of SASA to the whole surface area of each N-substituent during each MD run is plotted. The average during MD run is shown above each plot.
Table S2  Chemical structures of substituents at R₁, R₃, and R₅ and Kᵢ values of compounds S₁–S₇.

| Compound | R₁ | R₃ | R₅ | Kᵢ (µM) |
|----------|----|----|----|---------|
| S₁       |    |    |    | 22.3    |
| S₂       |    |    |    | 6.0     |
| S₃       |    |    |    | 24.9    |
| S₄       |    |    |    | 2.7     |
| S₅       |    |    |    | 1.9     |
| S₆       |    |    |    | 10.6    |
| S₇       |    |    |    | 23.8    |

*The Kᵢ values were determined by a competitive fluorescence polarization assay.*
Fig. S3  Inhibitory curves of 2–4 against the interaction between fluorescently labeled PMI peptide and MDM2 generated from a competitive FP assay. Error bars represent standard deviations of triplicates. Parameters obtained from the curve fitting (Hill equation: \( y = y_{\text{max}} - y_{\text{max}}/(1 + (IC_{50}/x)^n) \)) of the acquired data are shown in each graph.
Fig. S4. Inhibitory curves of S1–S7 against the interaction between fluorescently labeled PMI peptide and MDM2 generated from a competitive FP assay. Error bars represent standard deviations of triplicates. Parameters obtained from the curve fitting (Hill equation: $y = y_{\text{max}} - y_{\text{max}}/(1 + (\text{IC}_{50}/x)^n)$) of the acquired data are shown in each graph.
Table S3  Chemical structures of substituents at $R_2$ and $R_4$, and $P_e$ values of compound 4–9 determined by PAMPA.

| Compound | $R_2$, $R_4$ | $P_e$ ($\times 10^6$ cm/s) |
|----------|--------------|-----------------------------|
| 4        | (Me)         | 0.14 ± 0.03                 |
| 5        | (Et)         | 0.34 ± 0.02                 |
| 6        | (nPr)        | n.d.*                       |
| 7        | (nBu)        | n.d.*                       |
| 8        | (Moe)        | 0.22 ± 0.07                 |
| 9        | (Hoe)        | 0.14 ± 0.05                 |

*aNot determined due to the low water solubility in aqueous solution.*
Fig. S5  Inhibitory curves of 5, 8, and 9 against the interaction between fluorescently labeled PMI peptide and MDM2 generated from a competitive FP assay. Error bars represent standard deviations of triplicates. Parameters obtained from the curve fitting (Hill equation: \( y = y_{\text{max}} - \frac{y_{\text{max}}}{1 + (IC_{50}/x)^n} \)) of the acquired data are shown in each graph.
Fig. S6  CD spectra of 1–5, 8, and 9. CD spectra were recorded at 25°C with 50 µM solution of each oligomer in 20 mM phosphate buffer (pH 7.4) containing 5% methanol. The Y-axis was normalized to molar ellipticity per residue.
**Fig. S7**  ROESY spectrum of oligo-NSA 1-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. NOE peaks that support rotational restriction about backbone dihedral angles, $\phi$ and $\psi$, are shown with dashed lines in the spectrum. Protons that are suggested in proximity are shown with red and blue arrows in the chemical structure.
**Fig. S8** ROESY spectrum of oligo-NSA 5-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. NOE peaks that support rotational restriction about backbone dihedral angles, $\varphi$ and $\psi$, are shown with dashed lines in the spectrum. Protons that are suggested in proximity are shown with red and blue arrows in the chemical structure.
Fig. S9  $^1$H NMR spectrum of 1-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. Peaks were assigned with the aid of COSY, $^{13}$C NMR, HMQC, and HMBC spectra.
Fig. S10  COSY spectrum of 1-Pip. Spectrum was recorded in CDCl₃ at 25°C. Cross peaks that support assignment of ¹H NMR are shown with dashed lines.
Fig. S11  $^{13}$C NMR spectrum of 1-Pip. Spectrum was recorded in CDCl$_3$ at 25°C.
Fig. S12  HMOC spectrum of 1-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. Cross peaks that support assignment of $^1$H NMR are shown with dashed lines.
Fig. S13. HMBC spectrum of 1-Pip. Spectrum was recorded in CDCl₃ at 25°C. Cross peaks that support assignment of ¹H NMR are shown with dashed lines.
Fig. S14  $^1$H NMR spectrum of 5-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. Peaks were assigned with the aid of COSY, $^{13}$C NMR, HMQC, and HMBC spectra.
Fig. S15 COSY spectrum of 5-Pip. Spectrum was recorded in CDCl₃ at 25°C. Cross peaks that support assignment of ¹H NMR are shown with dashed lines.
**Fig. S16** \(^{13}\)C spectrum of 5-Pip. Spectrum was recorded in CDCl\(_3\) at 25°C.
Fig. S17  HMQC spectrum of 5-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. Cross peaks that support assignment of $^1$H NMR are shown with dashed lines.
Fig. S18  HMBC spectrum of 5-Pip. Spectrum was recorded in CDCl$_3$ at 25°C. Cross peaks that support assignment of $^1$H NMR are shown with dashed lines.
Fig. S19  Clustering results of the MD simulations of oligo-NSA 1 and 5. Frames from five runs were combined and the total frames were subjected to the clustering. (a, b) The generated clusters of 1 and 5 and the number of members classified to each cluster. The number of a representative frame is also shown for each cluster. Conformers corresponding to the representative frames of top 5 clusters are overlaid in Fig. 5d and e. (c, d) Each frame was colored using the color of the cluster shown in (a) and (b).
**Fig. S20**  RMSD values of α-carbons of oligo-NSA 1 and 5 from the initial structures during each MD run are plotted.
Fig. S21  Distributions of $\phi$ and $\psi$ angles of 1 and 5 during MD simulations. The distribution of 1st–4th residues are shown. The distribution of 5th residues are not shown because C terminal amide is not substituted, and 5th residues do not experience rotational restriction of $\phi$ and $\psi$ angles by pseudo-1,3-allylic strains.
Fig. S2  Clustering results of the MD simulations of oligo-NSA 1 and 5 in complex with MDM2. Frames from five runs were combined and the total frames were subjected to the clustering. (a, b) The generated clusters and the number of members classified to each cluster. The number of a representative frame is also shown for each cluster. (c, d) Each frame was colored using the color of the cluster shown in (a) and (b).
**Fig. S23**  Clustering results of MD simulations of oligo-NSA 1 and 5 in complex with MDM2. (a, b) Structures corresponding to the representative frames of top 5 clusters are overlaid. (c, d) The structures of oligo-NSA 1 and 5 in (a) and (b) were extracted.
Fig. S24  RMSD values of α-carbons of oligo-NSA 1 (a) or 5 (b) and MDM2 from the initial structures during each MD run are plotted.
Fig. S25  Ratio of SASA to the whole surface area of each N-substituent during each MD run is plotted. The average during MD run is shown above each plot.
**Fig. S26** Intracellular protein levels of p53, p21, and β-actin analyzed by western blotting. SJS-A-1 cells were treated with oligo-NSA or Nutlin-3a at the indicated concentrations for 8 h. (a, b) Results of two of three independent experiments are shown. Another result is shown in Fig. 6a.
Fig. S27  Uncropped images of Fig. 6a and S26a and b.
Fig. S28  Inhibitory curves of 5-Rv against the interaction between fluorescently labeled PMI peptide and MDM2 generated from competitive FP assay. Error bars represent standard deviations of triplicates. Parameters were obtained from the curve fitting (Hill equation: $y = y_{\text{max}} - y_{\text{max}}/(1 + (\text{IC}_{50}/x)^n)$) of the acquired data.
Fig. S29 Measurements of apoptotic SJSA-1 cells by staining with Annexin V-FITC and propidium iodide (PI) (Experiment 1/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SJSA-1 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S30 Measurements of apoptotic SJSA-1 cells by staining with Annexin V-FITC and PI (Experiment 2/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SJSA-1 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S31  Measurements of apoptotic SJSA-1 cells by staining with Annexin V-FITC and PI (Experiment 3/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SJSA-1 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S32  Measurements of apoptotic SW480 cells by staining with Annexin V-FITC and PI (Experiment 1/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SW480 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S33  Measurements of apoptotic SW480 cells by staining with Annexin V-FITC and PI (Experiment 2/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SW480 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S34  Measurements of apoptotic SW480 cells by staining with Annexin V-FITC and PI (Experiment 3/3). After the treatment with 0, 2.5, 5, or 10 µM 1, 5, 5-Rv, or Nutlin-3a for 48 h, SW480 cells were labeled with Annexin-V and PI, and analyzed by flow cytometry. Shown is one of the results of three independent experiments.
Fig. S35  Intracellular protein levels of p53, p21, and β-actin analyzed by Western blotting. SW480 cells were treated with oligo-NSA or Nutlin-3a at the indicated concentrations for 8 h. (a–c) Results of three independent experiments are shown. As a control, SJSA-1 cells were also treated with vehicle or Nutlin-3a and analyzed.
Fig. S36  Uncropped images of Fig. S35.
Fig. S37  HPLC chromatograms of compound 1, 1-Pip, 2–4, 5, 5-Pip, 5-Rv, and 6–9 after purification. Compounds were monitored at 220 nm.
Fig. S38  HPLC chromatograms of compound S1–S7 and Flu-PMI (the fluorescence probe used for FP assay) after purification. Compounds were monitored at 220 nm.
Fig. S39  Binding curves of Fluorescently labeled PMI peptide and MDM2 generated from FP assay. Error bars represent standard deviations of triplicates. Parameters obtained from the curve fitting (Hill equation: \( y = y_{\text{min}} + (y_{\text{max}} - y_{\text{min}})/(1 + (K_d/x)^n) \)) of the acquired data are shown.
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