Regular Article

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Elaboration of Non-naturally Occurring Helical Tripeptides as p53–MDM2/MDMX Interaction Inhibitors

Aoze Su,a Yuko Tabata,b Kiyono Aoki,b Akane Sada,b Ricko Ohki,*b Satoru Nagatoishi,c Kouhei Tsumoto,c,d Siyuan Wang,a Yuko Otani,a and Tomohiko Ohwada*a

aGraduate School of Pharmaceutical Sciences, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan; bLaboratory of Fundamental Oncology, National Cancer Center Research Institute; 5–1–1 Tsukiji, Chuo-ku, Tokyo 104–0045, Japan; cThe Institute of Medical Science, The University of Tokyo; 4–6–1 Shirokanedai, Minato-ku, Tokyo 108–8639, Japan; and dSchool of Engineering, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8656, Japan.

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Protein–protein interactions (PPIs) are often mediated by helical, strand and/or coil secondary structures at the interface regions. We previously showed that non-naturally occurring, stable helical trimers of bicyclic β-amino acids (Abh) with all-trans amide bonds can block the p53–MDM2/MDMX α-helix–helix interaction, which plays a role in regulating p53 function. Here, we conducted docking and molecular dynamics calculations to guide the structural optimization of our reported compounds, focusing on modifications of the C-terminal/N-terminal residues. We confirmed that the modified peptides directly bind to MDM2 by means of thermal shift assay, isothermal titration calorimetry, and enzyme-linked immunosorbent assay (ELISA) experiments. Biological activity assay in human osteosarcoma cell line SJSA-1, which has wild-type p53 and amplification of the Mdm2 gene, indicated that these peptides are membrane-permeable p53–MDM2/MDMX interaction antagonists that can rescue p53 function in the cells.

Key words helix mimic; protein–protein interaction; p53–MDM2 interaction; p53–MDMX interaction; docking simulation

Introduction
The interface regions of proteins involved in protein–protein interactions (PPIs) generally contain a mixture of approximately equal proportions of amino acid residues with helical, strand and coil structures. Among compounds that inhibit PPIs, stable, enzyme-resistant peptidomimetics containing artificial amino acids have attracted considerable attention due to their potential applications as research tools and candidate drugs. One potential target of PPI inhibitors is the p53–MDM2/MDMX α-helix–helix interaction, p53 functions as a tumor suppressor, and its PPI with MDM2 forms a MDM2–p53 interaction, which plays a role in regulating p53 function. Here, we conducted docking and molecular dynamics calculations to guide the structural optimization of our reported compounds, focusing on modifications of the C-terminal/N-terminal residues. We confirmed that the modified peptides directly bind to MDM2 by means of thermal shift assay, isothermal titration calorimetry, and enzyme-linked immunosorbent assay (ELISA) experiments. Biological activity assay in human osteosarcoma cell line SJSA-1, which has wild-type p53 and amplification of the Mdm2 gene, indicated that these peptides are membrane-permeable p53–MDM2/MDMX interaction antagonists that can rescue p53 function in the cells.

Crystal structure analysis has shown that MDM2 and MDMX have a deep binding pocket, to which p53 binds via a helical structure in its N-terminal domain. Three amino acid residues (Phe19, Trp23 and Leu26) of p53 extend into the concave binding surface and account for most of the binding energy. The MDMX binding site is similar to that of MDM2, except that two residues of MDMX, Met53 and Tyr99, extend into the pocket, making it narrower and slightly different in shape. The amino acid residues surrounding the MDM2 binding pocket are mostly hydrophobic.

As a part of our studies on enzyme-resistant peptidomimetics, we previously found that homooligomers of bicyclic β-amino acids (Abh-AA) can block the p53–MDM2 and p53–MDMX interactions (PPIs). In contrast to the natural right-handed α-helix with 3.6 residues/turn, these homoooligomers of (2R)-Abh-AA with all-trans amide bonds, that is, tAbh-AA, adopt a non-natural left-handed helical structure, with approximately 2.7 residues/turn. Although the tAbh-AA helix and α-helix have different numbers of residues per turn and different pitch, we hypothesized that tAbh-AA might interfere with PPIs involving an α-helix–helix complementary interaction. Our initial screening of a library of diverse Abh-AA oligomers revealed that homooligomers as short as the trimer, with a p-bromobenzoyl group at the N-terminal position, showed potent MDM2- and MDMX-antagonistic activities. This result suggested that the hydrophobic surface of the tAbh-AA helix fits well into the MDM2 pocket, blocking the interaction with p53. Due to their high hydrophobicity and relatively small molecular weight (M.W. approx. 550–650), these helical peptides are expected to penetrate the cell membrane, and might be effective as intracellular PPI modulators. This idea is supported by our previous finding that nitric oxide (NO)-releasing reagents with a similar bicyclic skeleton can enter the cell and activate the transient receptor potential A1 (TRP-A1) channel by transnitrosylating cysteine residues in the intracellular domain.

In this study, we set out to optimize the PPI-inhibitory activity of our previously reported compounds for the p53–MDM2/MDMX interaction by systematic modification of the N/C-terminal substituents on the basis of detailed docking studies and molecular dynamics simulation. Notably, we find that helix peptides as short as trimer can bind directly to MDM2 in an entropy-driven manner, and can restore intracellular p53 activity by binding at the interface region of p53–MDM2/MDMX, thereby antagonizing the interaction.

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Results and Discussion

Analysis of MDM2/MDMX-Binding Site and Binding Pose of tAbh-AA Peptides  The binding modes of lead peptides 1, 2, and 3 were investigated by docking simulation followed by molecular dynamics simulation of the peptide-protein complexes (Fig. 1a). The crystal structure of MDM2 bound to a peptidic inhibitor was used (PDB ID: 3jzs). Prior to docking simulation, potential binding sites of the MDM2 protein were predicted by Sitemap analysis and Mixed-solvent molecular dynamics (mixed-solvent MD).

Sitemap analysis detected several potential binding sites, including a binding site identical with that of p53 peptide analogues, as well as some small allosteric binding sites. Based on its size, shape and hydrophobicity, we considered that the p53 binding site is the only plausible binding region on MDM2 for our artificial helical peptides (Fig. 1c).

Evaluation of Binding Site While Sitemap analysis utilizes the surface character of a static protein structure, mixed-solvent MD, in contrast, considers not only the static surface, but also dynamic factors that would facilitate the competition of molecular probes with water molecules around the protein surface. In this analysis, the protein was surrounded with a layer of small-molecular probes (acetonitrile, isopropanol and dimethyl sulfoxide (DMSO)) and then sunk into a box of SPC water (the ratio of each probe to water was set at around 5%, n/n). Molecular dynamics simulation was carried out for a total of 10ns on mixed-solvent system in the OPLS3 force field. Water that was displaced by small-molecular probes, and that is considered as removable by binding of ligands, is displayed as spheres (Fig. 1d). The color gradient from red, to white, to blue represents free energies quantitatively from low to high. The highest density of low-energy interaction spots (red and white) appears around the cleft between MDM2 helix 2 and helix 4, which is the p53 binding site, supporting the idea that this is the most favorable binding site, because the low-energy spots are potential binding hotspots, in which the displacement of water by functional groups of ligands would be strongly stabilized during binding.

Within the broad hydrophobic pocket, we define the three sub-areas bound with the Phe19, Trp23 and Leu26 residues of p53 as Hotspots 1, 2 and 3, respectively (Fig. 1b). They account for most of the binding energy and were regarded as critical targets of structural design for inhibitory ligands.

Evaluation of Binding Pose Based on accessible protein models, molecular docking was carried out for the previously reported active compound, trimer 1. Plausible binding poses were generated, and 100ns molecular dynamics simulation was carried out on high-Glide-score docking poses. All poses obtained during the 100ns simulation period were clustered, and the putatively most stable binding pose of highest frequency was identified.

The binding pose thus obtained on MDM2 protein (Fig. 1e) indicates that the overall hydrophobicity of trimer 1 contributes to fitting in the hydrophobic binding pocket: the p-BrBz ring occupies Hotspot 1, mimicking the Phe19 residue of p53 and having a potential π-π interaction with Tyr67; the first and second bicyclic residues lie in Hotspots 2 and 3, respectively, with the C-terminus exposed to the solvent (Figs. 1b, e, f). The binding pose was stable during 100ns molecular dynamics simulation.

We previously found a length dependency of tAbh-AA...
oligomers for antagonistic activity towards both MDM2 and MDMX: the activity increases from dimer 2 to trimer 1 to tetramer 3. All three compounds show slightly stronger activity towards MDM2 than towards MDMX. In order to understand these findings, we generated possible binding poses of 2 and 3, using the same procedure as described above.

Dimer 2 bound to MDM2 occupies only two of the three hotspots of the hydrophobic pocket. The binding of 2 to MDM2 is not stable during even during 10 ns molecular dynamics simulation, during which the ligand’s position fluctuated within the pocket, dramatically changing its poses (Fig. 1g). This may account for the weak binding affinity of 2 to MDM2. A comparison of the unstable binding of 2 and the stable binding pose of 1 indicates that 2 is too small to fit well into the binding pocket and that a third Abh-AA residue is necessary to achieve stable binding to MDM2 protein. Tetra-
mer 3 shows a similar binding pose to 1 on MDM2 (Fig. 1h). It was found that the hydrophobic pocket of MDM2 became a little broader due to ligand-protein interaction during 100 ns molecular dynamics simulation. Further, the binding pose of 3 on MDMX is quite similar to that on MDM2, which is consistent with the similar inhibitory activities towards the p53 interaction with these two proteins. Although tetramer 3 showed good inhibitory activity, we selected trimer 1 as the lead compound for further modification for the following reasons: (1) poor solubility of 3 in water; (2) 1 and 3 occupy similar binding sites; and (3) 1 is the smallest molecule occupying all of Hotspots 1–3. Therefore, we anticipated the effect of changing the N- and C-termini of 1 would be easily recognizable.

Structural Modification Diversely substituted acyl groups were installed onto trimer 1, and the designed compounds (A-1–A-7, Fig. 2) were synthesized as illustrated in Chart 1. The activity of these compounds was evaluated with our reported enzyme-linked immunosorbent assay (ELISA) method, using compound Nutlin-317 and 1 as positive controls.

We found that A-1, A-3, A-5 and A-6 effectively inhibited the p53–MDM2 and p53–MDMX interactions (Fig. 3). Among them, A-3 was the most potent inhibitor at both 30 µM and 100 µM. The results indicate that aromatic substituents on the N-terminal (A-3, A-5, A-6 and 1) are generally advantageous for inhibitory activity. Comparing A-5 and 1, removal of the halogen substituent on N-terminal benzene ring did not cause loss of inhibitory activity. Bulky aromatic substituents such as naphthyl and p-t-butylphenyl (A-3 and A-6) increased the
activity, indicating that the N-terminal binding site regions of MDM2/X can accommodate bulky structures. The compound with a cyclohexyl group (A-1) had the comparable inhibitory activity to 1, whereas compounds with aliphatic acyl groups (A-2, A-4 and A-7) showed weaker inhibitory activity than 1 at both 30 and 100 µM.

The trimers appear to take similar binding poses with MDM2 protein in general, with the N-terminus binding to Hotspot 1 and the C-terminus located around Hotspot 3. The π–π interactions between N-terminal aromatic rings and the nearby Tyr67 residue of MDM2 contribute substantially to the binding affinity (Fig. 4a). In the case of A-1, CH–π interactions between the N-terminal cyclohexyl group and Tyr67 may also play a role (Fig. 4b).

Next, the C-terminal ester groups of three active compounds, 1, A-1 and A-3, were hydrolyzed to generate the corresponding free carboxylic acids, B-1, B-2 and B-3, respectively; Chart 1).

The Series B compounds showed improved solubility (see Experimental), with maintained or slightly increased inhibitory activity toward both p53–MDM2 and p53–MDMX interactions (Fig. 5). The IC_{50} values of Nutlin-3 for MDM2

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (µM) (on p53–MDM2)</th>
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<tbody>
<tr>
<td>Nutlin-3</td>
<td>1.0 (66)\textsuperscript{a}</td>
</tr>
<tr>
<td>1</td>
<td>579</td>
</tr>
<tr>
<td>A-1</td>
<td>218</td>
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<tr>
<td>A-3</td>
<td>357</td>
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<td>B-1</td>
<td>564</td>
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<td>B-2</td>
<td>123</td>
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<td>B-3</td>
<td>40</td>
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\textsuperscript{a} The IC_{50} value of Nutlin-3 (racemic) for GST-MDMX is shown in parentheses.

Fig. 6. (a) Docking Pose of B-3 Bound to MDM2
(b) Illustration of interactions between ligand B-3 and surrounding residues of MDM2 or solvent. White dots: solvent exposure. (Color figure can be accessed in the online version.)

Fig. 7. (a) DSF Experiment with MDM2 in the Presence or Absence of Peptides
(b) ITC experiment to examine the interaction between MDM2 and A-3. (Color figure can be accessed in the online version.)
and MDMX were also obtained (Table 1): IC$_{50}$ of Nutlin-3 for MDM2 was 1.0 µM whereas that for MDMX was 66 µM, respectively. This data suggested that Nutlin-3 showed relatively weaker inhibitory activity toward MDMX than that to MDM2. The IC$_{50}$ of the more active enantiomer of Nutlin-3 (Nutlin-3a), measured by means of a fluorescence polarization assay has been reported, and the IC$_{50}$ of Nutlin-3 for p53–MDM2 complex and that for p53–MDMX complex were reported to be 0.3 and 18.4 µM, respectively. The present IC$_{50}$ values were based on racemic Nutlin-3, which showed reasonably larger values than those of optical active Nutlin-3a. On the other hand, the rank order of Nutlin-3 with respect to MDM2 and MDMX was consistent with the reported trend. While B-3 is weaker than Nutlin-3, B-3 showed highest inhibitory activity on MDM2 (IC$_{50}$ 40 µM) among the helical peptides (Table 1). Although we did not carry out the absorption, distribution, metabolism and excretion (ADME) study of our compounds, all the compounds such as B-3 are compatible with the conventional experimental conditions such as in acidic/basic conditions. The compound B-3 does not decompose under the ambient temperature and in the presence of air.

Molecular docking study showed that compound B-3 takes similar docking poses with MDM2, and these were stable during 100 ns molecular dynamics simulation. A π–π interaction between the N-terminal naphthalene ring and the Tyr67 residue of MDM2 was also observed (Fig. 6a). The ionized C-terminus forms hydrogen bonds with nearby residue Lys51 and water molecules, which may stabilize the binding pose and direction (Fig. 6b).

Biophysical Interaction Analyses To validate binding of the peptides to the target protein MDM2, we used differential scanning fluorimetry (DSF) and isothermal titration calorimetry (ITC). TSA showed that peptides A-1, A-3, and B-3 reduced the $T_m$ of MDM2 protein from about 61°C to 60 or 59°C (Fig. 7a). These results indicate that the peptides interact with MDM2 protein and reduce its thermal stability. ITC measurements also indicated that the peptides bind to MDM2, although we could not quantitatively determine the binding affinity because the usable sample concentration was lower than the estimated binding affinity (Fig. 7b). The binding was found to be entropy-driven, suggesting that the interaction is mainly hydrophobic.

In Vivo Biological Activity Assay In order to evaluate the activity of the compounds in vivo, we chose the human osteosarcoma SJSA-1 cell line, which has wild-type p53 and amplification of the Mdm2 gene. It is well established that treatment of SJSA-1 cells with an Mdm2 inhibitor activates p53, leading to induction of p53 target genes. We therefore analyzed the induction of representative p53 target genes (PHLDA3 and p21) in SJSA-1 cells treated with the tripeptides. As expected, A-3 and B-3 showed stronger p53 activation abilities than 1. The induction of PHLDA3 was consistent with the activities determined by ELISA, and B-3 showed the highest activity. On the other hand, p21 was induced most strongly by A-3.

The finding that A-3 and B-3 show cellular activity at 30 µM, a concentration of the same order as the IC$_{50}$ value in the protein-level ELISA assay, suggests that these peptides are membrane-permeable and can enter the cells.

Expression of PHLDA3 (a) and p21 (b) in SJSA-1 cells treated with or without tripeptides. The cells were treated with the indicated compounds at 30 µM for 16h. PHLDA3 and p21 mRNA levels were determined by quantitative RT-PCR. Expression levels of the genes were normalized by the expression of 18S ribosomal RNA (rRNA). Error bars show mean values of three independent experiments with standard deviation (S.D.) *p < 0.05, **p < 0.005 and ***p < 0.001 (Student’s t-test).

Conclusion In this work we carried out systematic modification of the N/C-terminal substituents of our previously identified non-naturally occurring helical tripeptides in order to examine the structure–activity relationship for inhibition of p53–MDM2/MDMX interaction. We found that cyclohexyl and aromatic substituents at the N-terminal of (2R)-Abh-AA trimers increase the inhibitory activity by enhancing the binding to Hotspot 1 of the MDM2/MDMX binding pockets. Hydrolysis at the C-terminal improves both inhibitory activity and water solubility by enabling the formation of multiple hydrogen bonds with nearby residues.
bonds. DSF and ITC analyses confirmed that the peptides bind directly to MDM2 via an entropy-driven process. In SJSA-1 cells, the developed tripeptides activate p53, leading to induction of p53 target genes, thereby demonstrating their membrane-permeable character. We anticipate that changing the bridgehead substituent of these tripeptides may further increase the binding affinity via an enthalpy-driven process, such as hydrogen bonding.

**Experimental**

**General Synthetic Procedures** Melting points (mp) were determined with a Yanaco micro melting point apparatus without correction. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectra were recorded on a Bruker Avance400. Chemical shifts were calibrated with tetramethylsilane as an internal standard or with the solvent peak, and are shown in ppm (δ) values; coupling constants are shown in hertz (Hz). The following abbreviations are used: s = singlet, d = doublet, t = triplet, dd = double doublet, m = multiplet, and br s = broad singlet. An electron-spray ionization time-of-flight (ESI-TOF) mass spectrometer (Bruker micrOTOF-05) was used to obtain high-resolution mass spectra (HRMS). All commercially available compounds and solvents were used as received. All the tested compounds showed ≥95% purity on the basis of the NMR analysis.

**Synthesis**

**Compound A-4**

Trifluoroacetic acid (TFA) (1.5 mL) was added to a solution of tert-butoxycarbonyl (Boc)-((2R)-rAbh-AA)$_2$-O-Me (0.1970 g, 0.4222 mmol) in anhydrous CH$_2$Cl$_2$ (10.0 mL) at 0°C. The reaction mixture was stirred for 20 min at room temperature and then evaporated. The residue was washed with 10% Na$_2$CO$_3$ aqueous solution and extracted with EtOAc. The organic layer was dried over Na$_2$SO$_4$ and the solvent was evaporated. The residue was added to a solution of Boc-(2R)-Abh-AA-OH (0.1205 g, 0.4222 mmol), HATU (0.2408 g, 0.6333 mmol) and N,N-diisopropylethylamine (DIPEA) (0.22 mL, 1.2666 mmol) in anhydrous CH$_2$Cl$_2$ at 0°C, and the reaction mixture was allowed to warm to room temperature for 12 h under an Ar atmosphere, then washed with water, and extracted with CH$_2$Cl$_2$. The organic phase was dried over Na$_2$SO$_4$ and the solvent was evaporated. Column chromatography (n-hexane/ EtOAc = 2/1–1/1, v/v) of the residue afforded A-4 (29.5 mg, 81%) as a white solid.

mp 189–190°C

$^1$H-NMR (400 MHz, CDCl$_3$) δ: 7.537 (d, J = 8.8 Hz, 2H), 7.457 (d, J = 8.4 Hz, 2H), 6.423–6.400 (m, 1H), 4.582–4.559 (m, 1H), 4.516 (d, J = 10.4 Hz, 1H), 4.469 (d, J = 10.0 Hz, 1H), 4.387 (d, J = 10.4 Hz, 1H), 4.086–4.072 (m, 1H), 4.072 (d, J = 10.4 Hz, 1H), 3.706 (s, 3H), 3.506–3.467 (m, 1H), 3.427 (s, 3H), 3.406 (s, 3H), 3.396 (s, 3H), 3.346–3.306 (m, 1H), 3.183–3.144 (m, 1H), 2.133–1.599 (m, 17H), 1.485–1.415 (m, 1H).

$^{13}$C-NMR (100 MHz, CDCl$_3$) (some peaks were not detected due to the overlap of signals) δ: 173.48, 170.25, 169.78, 135.93, 131.63, 129.68, 125.17, 71.68, 71.52, 71.09, 62.16, 59.50, 59.25, 59.00, 58.76, 52.03, 47.83, 46.14, 46.06, 33.41, 29.76, 27.01.

TFA (0.5 mL) was added to a solution of A-4 (20.5 mg, 0.0316 mmol) in anhydrous CH$_2$Cl$_2$ (3 mL) at 0 °C. The reaction mixture was stirred for 10 min at 25 °C, and then evaporated. Et$_3$O (5 mL) was added to the residue and evaporated; this procedure was repeated 3 times. The obtained crude product was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL), and then Et$_3$N (11.0 µL, 0.0789 mmol) and acetyl chloride (2.7 µL, 0.0379 mmol) were added at 0 °C. The mixture was stirred for 9 h at 25 °C under an argon atmosphere, and then evaporated. The residue was washed with brine (20 mL), and extracted with EtOAc (20 mL × 2). The organic solution was dried over Na$_2$SO$_4$, and column chromatography (n-hexane/EtOAc = 1/2 to EtOAc 100%) then EtOAc/MeOH = 15:1, v/v) of the residue afforded A-2 (17.5 mg, 95%) as a white solid.

mp 72–74 °C

$^1$H-NMR

(400 MHz, CDCl$_3$) $\delta$: 4.596–4.574 (m, 1H), 4.552–4.529 (m, 1H), 4.478 (d, $J$ = 10.0 Hz, 1H), 4.352 (d, $J$ = 10.0 Hz, 1H), 4.044 (d, $J$ = 10.0 Hz, 1H), 3.830 (d, $J$ = 10.0 Hz, 1H), 3.732 (d, $J$ = 10.0 Hz, 1H), 3.692 (s, 3H), 3.441 (s, 3H), 3.384 (s, 3H), 3.379 (s, 3H), 3.351–3.284 (m, 2H), 3.154–3.125 (m, 1H), 2.038 (s, 3H), 2.082–1.418 (m, 18H).

$^{13}$C-NMR

(100 MHz, CDCl$_3$) (some peaks were not detected due to the overlap of signals) $\delta$: 173.45, 170.46, 170.25, 168.95, 72.61, 71.57, 71.03, 70.86, 59.41, 59.20, 58.80, 58.71, 51.98, 47.80, 46.11, 45.98, 33.48, 33.29, 29.76, 27.01.

HRMS


Compound A-3

TFA (0.5 mL) was added to a solution of A-4 (16.0 mg, 0.0253 mmol) in anhydrous CH$_2$Cl$_2$ (3 mL) at 0 °C. The reaction mixture was stirred for 10 min at 25 °C, and then evaporated. Et$_3$O (5 mL) was added to the residue and evaporated; this procedure was repeated 3 times. The obtained crude product was dissolved in anhydrous CH$_2$Cl$_2$ (2 mL), and then Et$_3$N (8.8 µL, 0.0789 mmol) and naphthoyl chloride (5.8 mg, 0.0303 mmol) were added at 0 °C. The mixture was stirred for 9 h at 25 °C under argon atmosphere, and then evaporated. The residue was washed with brine (20 mL) and extracted with EtOAc (20 mL × 2). The organic solution was dried over Na$_2$SO$_4$, and column chromatography (n-hexane/EtOAc = 1/2) of the residue afforded A-3 (16.8 mg, 95%) as a white amorphous solid.

$^1$H-NMR

(400 MHz, CDCl$_3$) $\delta$: 8.091 (s, 1H), 7.885–7.835 (m, 3H), 7.661–7.635 (m, 1H), 7.553–7.508 (m, 2H), 4.620–4.611 (m, 2H, overlapped), 4.543–4.498 (m, 1H), 4.474 (d, $J$ = 10.0 Hz, 1H), 4.208–4.197 (m, 1H), 4.062 (d, $J$ = 10.0 Hz, 1H), 4.022–4.010 (m, 1H), 3.948–3.933 (m, 1H), 3.798–3.779 (m, 1H), 3.788–3.770 (m, 1H), 3.739–3.728 (m, 1H), 3.696–3.680 (m, 1H), 3.670–3.653 (m, 1H), 3.064–3.042 (m, 1H), 2.978–2.950 (m, 1H), 2.917–2.894 (m, 1H), 2.853–2.832 (m, 2H), 2.790–2.754 (m, 1H), 2.733–2.704 (m, 1H), 2.685–2.652 (m, 1H), 2.640–2.614 (m, 1H), 2.604–2.572 (m, 1H), 2.517–2.498 (m, 1H), 2.432–2.410 (m, 1H), 2.382–2.369 (m, 1H), 2.350–2.330 (m, 1H), 2.319–2.305 (m, 1H), 2.047–2.028 (m, 1H), 1.983–1.961 (m, 1H), 1.739–1.724 (m, 1H), 1.705–1.684 (m, 1H), 1.651–1.634 (m, 1H), 1.600–1.583 (m, 1H), 1.542–1.520 (m, 1H), 1.490–1.466 (m, 1H), 1.445–1.421 (m, 1H), 1.396–1.377 (m, 1H), 1.364–1.346 (m, 1H), 1.333–1.315 (m, 1H), 1.303–1.283 (m, 1H), 1.276–1.256 (m, 1H).

Found: 644.3905.
(d, $J = 10.0$ Hz, 1H), 3.760 (d, $J = 10.0$ Hz, 1H), 3.704 (s, 3H), 3.563–3.512 (m, 1H), 3.446 (s, 3H), 3.412 (s, 3H), 3.397 (s, 3H), 3.387–3.301 (m, 1H), 3.186–3.134 (m, 1H), 2.097–1.606 (m, 17H), 1.469–1.418 (m, 1H).

$^{13}$C-NMR

(100 MHz, CDCl$_3$) (some peaks were not detected due to the overlap of signals) $\delta$: 173.47, 171.28, 171.06, 170.37, 170.28, 134.44, 134.33, 132.72, 128.86, 128.22, 128.10, 127.87, 127.41, 126.70, 125.03, 71.65, 71.47, 71.06, 62.22, 60.53, 59.22, 59.01, 58.74, 51.99, 47.83, 33.47, 29.78, 27.80.

HRMS


Compound A-5

TFA (1.0 mL) was added to a solution of A-4 (20.0 mg, 0.0316 mmol) in anhydrous CH$_2$Cl$_2$ (2 mL) at 0 °C. The reaction mixture was stirred for 15 min at 25 °C, and then evaporated. Et$_2$O (5 mL) was added to the residue and evaporated; this procedure was repeated 3 times. The obtained crude product was dissolved in anhydrous CH$_2$Cl$_2$ (2 mL), and then Et$_3$N (11 µL, 0.0794 mmol) and benzoyl chloride (5.3 mg, 0.0379 mmol) were added at 0 °C. The mixture was stirred for 9 h at 25 °C under an argon atmosphere, and then evaporated. The residue was washed with brine (20 mL), and extracted with EtOAc (20 mL $\times$ 2). The organic solution was dried over Na$_2$SO$_4$, and column chromatography ($n$-hexane/EtOAc = 1/1) of the residue afforded A-5 (15.0 mg, 75%) as a white amorphous solid.

$^1$H-NMR

(400 MHz, CDCl$_3$) $\delta$: 7.587–7.421 (m, 3H), 7.458 (d, $J = 8.4$ Hz, 2H), 4.621–4.601 (m, 1H), 4.581–4.561 (m, 1H), 4.512 (d, $J = 10.4$ Hz, 1H), 4.468 (d, $J = 10.0$ Hz, 1H), 4.388 (d, $J = 10.4$ Hz, 1H), 4.088–4.071 (m, 1H), 4.072 (d, $J = 10.4$ Hz, 1H), 3.702 (s, 3H), 3.501–3.467 (m, 1H), 3.422 (s, 3H), 3.401 (s, 3H), 3.398 (s, 3H), 3.347–3.302 (m, 1H), 3.181–3.142 (m, 1H), 2.133–1.591 (m, 17H), 1.484–1.412 (m, 1H).

$^{13}$C-NMR

(100 MHz, CDCl$_3$) (some peaks were not detected due to the overlap of signals) $\delta$: 173.51, 170.30, 169.80, 135.90, 131.61, 129.66, 125.10, 71.72, 71.10, 71.00, 62.19, 59.52, 59.29, 59.10, 58.76, 52.00, 47.90, 46.12, 46.06, 34.21, 33.42, 31.30, 29.78, 27.10.

HRMS


Compound A-6

TFA (0.5 mL) was added to a solution of A-4 (20.2 mg, 0.0319 mmol) in anhydrous CH$_2$Cl$_2$ (1 mL) at 0 °C. The reaction mixture was stirred for 15 min at 25 °C, and then evaporated. Et$_2$O (5 mL) was added to the residue and evaporated; this procedure was repeated 3 times. The obtained crude product was dissolved in anhydrous CH$_2$Cl$_2$ (2 mL), and then Et$_3$N (11 µL, 0.0794 mmol) and 4-tert-butylbenzoyl chloride (7.5 mg, 0.0383 mmol) were added at 0 °C. The mixture was stirred for 9 h at 25 °C under an argon atmosphere, and then evaporated. The residue was washed with brine (20 mL), and extracted with EtOAc (20 mL $\times$ 2). The organic solution was dried over Na$_2$SO$_4$, and column chromatography ($n$-hexane/EtOAc = 1/1) of the residue afforded A-6 (18.2 mg, 82%) as a white solid. mp 151–154 °C

$^1$H-NMR

(400 MHz, CDCl$_3$) $\delta$: 7.570–7.411 (m, 2H), 7.457 (d, $J = 8.4$ Hz, 2H), 4.631–4.611 (m, 1H), 4.571–4.551 (m, 1H), 4.512 (d, $J = 10.4$ Hz, 1H), 4.469 (d, $J = 10.0$ Hz, 1H), 4.398 (d, $J = 10.4$ Hz, 1H), 4.078–4.051 (m, 1H), 4.062 (d, $J = 10.4$ Hz, 1H), 3.706 (s, 3H), 3.501–3.467 (m, 1H), 3.421 (s, 3H), 3.406 (s, 3H), 3.399 (s, 3H), 3.347–3.302 (m, 1H), 3.181–3.142 (m, 1H), 2.138–1.592 (m, 17H), 1.474–1.410 (m, 1H), 1.352 (s, 9H).

$^{13}$C-NMR

(100 MHz, CDCl$_3$) (some peaks were not detected due to the overlap of signals) $\delta$: 177.50, 170.51, 169.90, 135.90, 131.21, 129.66, 125.10, 71.72, 71.10, 71.00, 62.19, 59.52, 59.29, 59.10, 58.76, 52.00, 47.90, 46.12, 46.06, 34.21, 33.42, 31.30, 29.78, 27.10.

HRMS


Compound A-7

TFA (0.5 mL) was added to a solution of A-4 (21.3 mg, 0.0336 mmol) in anhydrous CH$_2$Cl$_2$ (2 mL) at 0 °C. The reaction mixture was stirred for 15 min at 25 °C, and then evaporated. Et$_2$O (5 mL) was added to the residue and evaporated;
this procedure was repeated 3 times. The obtained crude product was dissolved in anhydrous CH₂Cl₂ (2 mL), and then Et₃N (11 µL, 0.0794 mmol) and heptanoyl chloride (7 µL, 0.0403 mmol) were added at 0 °C. The mixture was stirred for 9 h at 25 °C under an argon atmosphere, and then evaporated. The residue was washed with brine (20 mL), and extracted with EtOAc (20 mL × 2). The organic solution was dried over Na₂SO₄, and column chromatography (n-hexane/ethyl acetate = 1/1) of the residue afforded A-7 (17.7 mg, 82%) as a white solid.

**mp 92–93 °C**

**1H-NMR** (400 MHz, CDCl₃) δ: 4.583–4.571 (m, 2H), 4.477 (brd, J = 9.6 Hz, 1H), 4.350 (d, J = 10.0 Hz, 1H), 4.207 (m, 1H), 4.046 (d, J = 10.0 Hz, 1H), 3.837 (d, J = 10.0 Hz, 1H), 3.737 (d, J = 10.0 Hz, 1H), 3.691 (s, 3H), 3.421 (s, 3H), 3.381 (s, 3H), 3.347–3.263 (m, 2H), 3.149–3.120 (m, 1H), 2.275–2.263 (m, 2H), 2.081–1.807 (m, 9H), 1.743–1.419 (m, 1H), 1.317–1.240 (m, 6H), 0.864 (t, J = 6.8 Hz, 3H).

**13C-NMR** (100 MHz, CDCl₃) (some peaks were not detected due to the signal broadening and overlaps) δ: 173.28, 171.70, 170.25, 170.09, 72.52, 71.39, 70.86, 70.80, 59.25, 59.04, 58.84, 58.83, 58.66, 58.56, 51.81, 47.67, 45.97, 45.83, 35.83, 33.26, 33.20, 31.60, 30.62, 30.32, 29.66, 29.41, 29.11, 27.73, 27.15, 26.48, 25.08, 22.49, 14.01.


**Compound B-1**

A solution of LiOH·H₂O (10.0 mg, 0.24 mmol) in water (1.5 mL) was added to a solution of 1 (18.5 mg, 0.03 mmol) in tetrahydrofuran (THF) (3.0 mL). Then two drops of methanol were added. The reaction mixture was stirred at 25 °C for 10 h, then the reaction was quenched by adding 5% KHSO₄ aqueous solution (20 mL). The mixture was extracted with CHCl₃ and the organic solution was dried over Na₂SO₄ and evaporated to afford B-1 (18.0 mg, 99%) as a white amorphous solid.

**1H-NMR** (400 MHz, CDCl₃) δ: 7.521 (d, J = 8.6 Hz, 2H), 7.436 (d, J = 8.6 Hz, 2H), 4.628–4.582 (m, 2H), 4.523–4.445 (m, 2H), 4.349 (brd, J = 10.0 Hz, 1H), 4.085 (m, 1H), 3.946 (d, J = 10.0 Hz, 1H), 3.718 (d, J = 8.6 Hz, 2H), 3.691 (s, 3H), 3.488–3.459 (m, 1H), 3.420 (s, 3H), 3.405 (s, 3H), 3.346–3.309 (m, 1H), 3.191–3.155 (m, 1H), 2.176–1.449 (m, 18H).

**13C-NMR** (100 MHz, CDCl₃) δ: 174.33, 170.35, 170.08, 169.56, 135.65, 131.45, 129.47, 125.01, 74.25, 71.48, 71.33, 70.61, 69.70, 62.00, 59.57, 59.42, 59.30, 58.80, 58.59, 58.25, 46.17, 45.97, 45.78, 45.60, 45.42, 44.10, 34.40, 34.05, 33.36, 33.14, 30.72, 29.56, 29.08, 27.69, 26.83.


**Compound B-2**

A solution of LiOH·H₂O (4.5 mg, 0.11 mmol) in water (1.0 mL) was added to a solution of A-1 (9.13 mg, 0.013 mmol) in THF (2.0 mL). Then two drops of methanol were added to the mixture. The reaction mixture was stirred at 25 °C for 10 h, then the reaction was quenched by adding 5% KHSO₄ aqueous solution (20 mL). The mixture was extracted with CHCl₃ and the organic solution was dried over Na₂SO₄ and evaporated to afford B-2 (7.85 mg, 88%) as a white solid.

**mp 87–90 °C**

**1H-NMR** (400 MHz, CDCl₃) δ: 4.587–4.577 (m, 2H), 4.497–4.472 (m, 2H), 4.374–4.349 (m, 2H), 4.285–4.268 (m, 2H), 3.843–3.818 (d, 1H), 3.744–3.719 (d, 1H), 3.530 (s, 3H), 3.423 (s, 3H), 3.389 (s, 3H), 3.299–3.262 (m, 2H), 3.171–3.143 (m, 1H), 2.302–2.264 (m, 1H), 2.170–1.193 (m, 27H).

**13C-NMR** (100 MHz, CDCl₃) δ: 174.59, 173.19, 170.56, 170.62, 74.60, 71.70, 71.53, 71.37, 70.98, 69.72, 59.60, 59.38, 58.86, 58.77, 58.59, 58.36, 53.92, 50.28, 46.19, 46.05, 44.10, 34.21, 33.90, 33.49, 30.75, 29.96, 29.83, 29.59, 29.39, 28.03, 26.70, 26.07.


**Compound B-3**

A solution of LiOH·H₂O (4.4 mg, 0.105 mmol) in water (1.0 mL) was added to a solution of A-3 (8.87 mg, 0.013 mmol) in tetrahydrofuran (THF) (2.0 mL). Then two drops of methanol were added to the mixture. The reaction mixture was stirred at 25 °C for 10 h, and the reaction was quenched by adding 5% KHSO₄ aqueous solution (20 mL). The mixture was extracted with CHCl₃ and the organic solution was dried over Na₂SO₄ and evaporated to
afford B-3 (6.89 mg, 83%) as a white amorphous solid.

1H-NMR

(400 MHz, CDCl3) δ: 8.092 (s, 1H), 7.888–7.841 (m, 3H), 7.649 (dd, J = 8.2, 1.4 Hz, 1H), 7.547–7.515 (m, 2H), 4.646–4.624 (m, 2H), 4.559–4.502 (m, 3H), 4.437 (d, J = 10.0 Hz, 1H), 4.292–4.275 (m, 1H), 4.215–4.204 (m, 1H), 4.018 (d, J = 10.4 Hz 1H), 3.738 (d, J = 10.0 Hz, 1H), 3.571 (s, 3H), 3.540–3.529 (m, 1H), 3.451 (s, 3H), 3.421 (s, 3H), 3.387–3.327 (m, 1H), 3.182–3.158 (m, 1H), 2.44 (dd, J = 12.4, 4.0 Hz, 1H), 1.724–1.400 (m, 17H).

13C-NMR

(100 MHz, CDCl3): 172.43, 170.90, 170.38, 170.27, 134.30, 134.09, 132.55, 128.71, 128.08, 127.97, 127.72, 127.29, 126.57, 124.84, 74.93, 71.47, 71.33, 70.81, 69.38, 65.56, 62.06, 59.55, 59.24, 59.03, 58.87, 58.63, 58.12, 50.68, 46.02, 34.25, 33.34, 33.04, 31.91, 30.68, 29.68, 28.53, 26.96, 26.59.

HRMS


Solubility

Among methyl ester compounds, the solubility of I and A-3 in DMSO was so low not to allow making 100 mM stock solutions in DMSO. Instead, we can make 100 mM stock solutions and in cases of other compounds including the carboxylic acid derivatives such as B-1, B-2, and B-3 can make 100 mM stock solutions in DMSO. Therefore, the final concentration of DMSO is 1% (v/v) in the cases of I and A-3, and in cases of other compounds, the final DMSO concentration is 0.1% (v/v) in our biological studies.

Biological Studies

Expression and Purification of Glutathione S-Transferase (GST)-Fusion Proteins

GST fusion construct of MDM2 (amino acid residues 1-150) was prepared by PCR tagging of the cDNAs with BamHI and Xhol sites at the 5′ and 3′ ends, respectively, and subcloned into pGEX-6P-1 vector (Amer sham Pharmacia, Tokyo, Japan). Constructs were expressed in Escherichia coli (BL21-Gold (DE3), Stratagene, CA, U.S.A.) and purified from cell lysates using glutathione-Sepharose 4B beads (Amer sham Pharmacia), eluted with glutathione buffer (100 mM Tris–HCl pH 8.5, 50 mM NaCl, 1 mM ethylene-aminetetraacetic acid (EDTA), 20 mM glutathione).

Biochemical Activity Assays by ELISA

The biological activities of synthesized compounds were evaluated by ELISA using GST-tagged MDM2 and MDMX proteins. GST-MDM2 (80 or 40 µg/mL in phosphate-buffered saline (PBS)) or MDMX (30 µg/mL in PBS) was coated on a 96-well plate (96 Well ELISA Microplate, Half Area, 675061, Greiner) overnight. Subsequently, test compounds (30 or 100 µg/mL in PBS) were added and incubated for 1 h, followed by addition of p53 (0.4 µg/mL in PBS). After 2-hour incubation, p53 bound to MDM2 or MDMX was detected by using anti-p53 antibody (mouse, monoclonal, Calbiochem (Merck, U.K.) as the first antibody and anti-mouse immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked whole antibody #NA931V (GE Healthcare, IL, U.S.A.) as the second antibody. The substrate ABT-S was then added together with hydrogen peroxide. Finally, the absorbance at 405 nm of each well was measured with a spectrophotometer, and the inhibitory ratio of each compound was calculated. Nutlin-3, reported by Vassilev et al.,17 was used as the positive control.

In Vivo Biological Activity Assay

SJSA-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Where indicated, cells were treated with the compounds for 16h. RNA was prepared using an RNeasy Mini kit (QIAGEN, Hilden, Germany). Reverse transcription was carried out using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. Total RNA was used for reverse transcription. Reverse-transcribed cDNAs were subjected to real-time PCR, which was performed with a CFX96 Touch Real-Time PCR System (Bio-Rad, CA, U.S.A.). For the detection of PHLDA3 and IRS rRNA, a PrimeTime qPCR Assay from Integrated DNA Technologies was used. For the detection of p21, TaqMan probe (Hs00355782_m1) from Applied Biosystems was used.

Thermal Shift Assay

Thermal shift assay was performed with a CFX Connect Real-Time PCR Detection System (Bio-Rad). The protein concentration was set at 0.2 mg/mL, and SYPRO-Orange was used as the fluorescent detection dye. These experiments were conducted in PBS buffer.

ITC Experiments

ITC experiments were carried out using a MicroCal iTC200 (Malvern Instruments, U.K.). The peptide solution (500 µM) was titrated into a sample cell containing a solution of MDM2 protein (17 µM). These experiments were conducted in PBS buffer at 25 °C.

Molecular Docking

The co-crystal structure of human MDM2 liganded with a 12mer peptide inhibitor (PDB ID: 3Z3S)12 was preprocessed using Protein Preparation Wizard in the Maestro2017-4 suite of programs (Schrödinger, LLC, U.S.A.). The embedded ligand was removed, and the target ligand was docked using Glide in the standard precision (SP) mode. The poses were sorted in order of docking score up to 30 poses. All 30 poses were structurally refined by means of molecular dynamics simulations. The ligand–protein bound system was solvated with water (SPC water model) with the OPLS3 force field.16 The charge was neutralized by adding 6 Cl− buffered with 0.15 M NaCl and 1 M Cl−. Molecular dynamic simulation of the whole system (NPT ensemble, temperature 300 K, pressure 1.013 bar (1 atm)) was conducted for 10 ns with the Desmond program. The structure that gave the best MM-GBSA ΔGbind value among the 30 poses at 10 ns is shown in Fig. 1 as a typical structure. Docking scores and MM/GBSA of (2R)-rAhb homooligomer ligands were generated by Glide.

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Conflict of Interest

The authors declare no conflict of interest.

References


