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# 1,4,5-Trisubstituted Imidazole-Based p53- MDM2/MDMX Antagonists with Aliphatic Linkers for Conjugation with Biological Carriers

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## ABSTRACT

The tumor suppressor protein p53, the “guardian of the genome”, is inactivated in nearly all cancer types by mutations in the *TP53* gene or by overexpression of its negative regulators, oncoproteins MDM2/MDMX. Recovery of p53 function by disrupting the p53-MDM2/MDMX interaction using small-molecule antagonists could provide an efficient non-genotoxic anticancer therapy. Here we present the syntheses, activities and crystal structures of the p53-MDM2/MDMX inhibitors based on the 1,4,5-trisubstituted imidazole scaffold which are appended with aliphatic linkers that enable coupling to bioactive carriers. The compounds have favorable properties at both biochemical and cellular levels. The most effective compound **19** is a tight binder of MDM2 and activates p53 in cancer cells that express the wild type p53, leading to cell cycle arrest and growth inhibition. Crystal structures reveal that compound **19** induces MDM2 dimerization *via* the aliphatic linker. This unique dimerization-binding mode opens new prospects for the optimization of the p53-MDM2/MDMX inhibitors and conjugation with bioactive carriers.

## INTRODUCTION

The tumor suppressor p53 is inactivated in the majority of human cancers, which is accomplished either by mutations in the *TP53* gene or by overexpression of p53 negative regulators, primarily MDM2 and/or MDMX.<sup>1-4</sup> Restoration of the function of p53 has been suggested as a promising strategy against cancer.<sup>1,2,5</sup> Mutations in the p53-encoding gene are found in about half of all human cancers and may be combated by gene therapy.<sup>6</sup> In the remaining 50% of cancer cases, in which wild type p53 (p53<sup>wt</sup>) is present, restoration of its activity by disrupting the interaction with MDM2/X proteins could be achieved by the use of small-molecule antagonists of the MDM2/MDMX-p53 interaction. It has been shown that such antagonists induce p53 downstream proteins, including p21, which leads to the induction

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3 of the G0/G1 and G2/M cell cycle arrests and additionally induction of apoptosis in some  
4 cancer cell types.<sup>7-10</sup> Such an approach promises a broad range non-genotoxic therapy.<sup>1,11</sup>  
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8 Several classes of small-molecule MDM2 antagonists entered clinical trials.<sup>3,12-15</sup> However,  
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10 there are many challenges before entering clinical practice, mainly concerning  
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12 pharmacological properties, and a constant progress is needed to improve the potency of the  
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14 compounds and provide precise targeting of cancer cells.<sup>16-19</sup>  
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18 The interaction of the N-terminal domain of MDM2 and MDMX with the N-terminal  
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20 transactivation domain of p53 is mediated by three key side chains of p53  $\alpha$ -helix: Phe19,  
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22 Trp23 and Leu26, which occupy hydrophobic subpockets on the MDM2 or MDMX  
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24 surface.<sup>20,21</sup> These interactions form a three finger pharmacophore, which is present in the  
25  
26 most of the currently available small-molecule MDM2 inhibitors.<sup>22,23</sup> Apart from this  
27  
28 canonical binding mode, several new approaches were recently proposed,<sup>24,25</sup> for instance  
29  
30 utilizing the transient states of MDM2 for the design of the small molecules occupying yet  
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32 another, fourth subpocket within MDM2 (inhibitor **YH300**, 3-(2-(tert-butylamino)-1-(N-(4-  
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34 (4-chlorobenzyloxy)benzyl)formamido)-2-oxoethyl)-6-chloro-1H-indole-2-carboxylic acid).<sup>26</sup>  
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38 A promising class of the p53-based stapled peptides have been recently proposed as another  
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40 novel group of antagonists.<sup>27,28</sup>  
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44 Previously, we reported a novel compound based on an imidazole scaffold (6-chloro-3-(1-  
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46 (4-chlorobenzyl)-4-phenyl-1H-imidazol-5-yl)-1H-indole-2-carboxylic acid, **WK23**, named  
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48 thereafter **1.1**) and capable of disrupting the MDM2-p53 interaction *in vitro*.<sup>29</sup> In the present  
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50 study, we characterize compound **1** (Table 1), a derivative of **1.1**. Compared to **1.1**, the  
51  
52 derivatives of **1** have stronger binding to MDM2 and the improved p53-activating and  
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54 growth-inhibitory activities in cell-based assays. We also report a series of modifications of **1**  
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56 with aliphatic linkers at the carboxylic group that is attached to the indole ring system  
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58 (Table 1). This further improves biological properties of the derivatives of **1**. Our structure-  
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3 based analysis suggests the potential use of such aliphatic groups for conjugation of the  
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6 compounds with biological carriers to improve solubility, stability and precise targeting to  
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8 malignant neoplasms.  
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## 10 11 12 13 RESULTS AND DISCUSSION

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15 **Optimization of Compound 1.** In a previous study we identified **1** as one of the most  
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17 potent derivatives of **1.1** in disrupting the p53/MDM2 interaction in *in vitro* biochemical  
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19 assays.<sup>29</sup> Here we evaluate the activity of **1** in the cells and optimize its p53-activation,  
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21 growth-inhibitory and cell-cycle arrest properties by decorating the **1** scaffold (Table 1).  
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25 Analyzing the co-crystal structure of **1.1** with MDM2, we identified position R<sub>2</sub> as a  
26  
27 potential site for compound expansion. The substituents at this position should point away  
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29 from the MDM2 molecule allowing for attachment of solubility/permeability enhancing  
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31 moieties or linkers for conjugation with targeting biological molecules, without significantly  
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33 affecting the affinity. Such assumptions were tested in compounds **13-19**.  
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37 **1** was obtained in the three-component (3CR) van Leusen reaction (Figure 1).<sup>30</sup> Position R<sub>2</sub>  
38  
39 was modified to obtain both amide (**2-13**) and ester (**14-19**) derivatives using classical  
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41 chemistry (SI Schemes S1-S2, SI Tables S3-S4). Amide derivatives included two kinds of  
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43 substituents, polar groups expected to improve the pharmacokinetic properties of **1** and  
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45 aliphatic moieties tested to evaluate if additional binding at the surface of MDM2 may be  
46  
47 obtained. Ester derivatives included aliphatic linkers containing terminal reactive group  
48  
49 envisioned for future conjugation with biological carriers/targeting molecules. The binding of  
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51 selected derivatives was initially tested by NMR titration<sup>31,32</sup> and affinities (K<sub>i</sub>) of all  
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53 evaluated derivatives were tested by fluorescence polarization (FP) assay (Table 1, plots  
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55 presented in SI Table S5).  
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3 Different modifications at R<sub>2</sub> position had different effects on the affinity towards MDM2,  
4 demonstrating that even though this substituent predictably should point away from the  
5 protein molecule, certain moieties were still poorly accepted. Modification with 4-  
6 morpholinopiperidinyl (**8**), 2-morpholinoethyl (**14**) or 6-succinyloxyhexyl (**16**) showed no  
7 negative effect on affinity. The binding of **3**, **4**, **6**, **18**, and **19** was slightly negatively affected,  
8 but the effect was negligible. The effect of other moieties was more pronounced with the  
9 affinities increasing above two digit nanomolar range (e.g. **2**, **5**, **7**, **9**, and **13**). Bulky  
10 substituents were not accepted at all at R<sub>2</sub> position (**10-12**). Both tested modification  
11 chemistries (amide vs ester) were compatible with MDM2 binding (e.g. **8** and **14**), although  
12 particular substituents favored certain chemistries as demonstrated by an order of magnitude  
13 better affinity of **14** compared to **13**. Since the modifications were introduced primarily with  
14 the aim of improving the effect of compounds on cells, all the derivatives which affinity was  
15 not significantly negatively affected were further evaluated in cellular assay as described  
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Potency of tested compounds towards MDMX followed the trend established for MDM2,  
although the affinities towards the former protein were around three orders of magnitude  
lower than those towards the latter as previously observed for **1.1**. **1** was the most potent  
MDMX binder with affinity (K<sub>i</sub>) of 3.15 μM. Clearly, despite significant homology of MDM2  
and MDMX, subtle differences in their p53 binding pockets<sup>33</sup> preclude optimization of **1**  
scaffold for concurrent binding to both those proteins.

Bista et al. have demonstrated that extending the Leu26-mimicking substituent within the  
three finger pharmacophore in the context of a 6-chloroindole scaffold opens an inducible  
noncanonical pocket by constraining Tyr100 in a position beneficial for binding larger  
substituents. Such four pocket binding mode was associated with improvement of affinity.<sup>26</sup>  
Here we tested if similar advantageous effect may be obtained within **1** scaffold. To this end

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3 we introduced elongated benzyl amines in position R<sub>1</sub>. These modifications (**21-24**) resulted  
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5 in significant reduction of affinity towards MDM2 demonstrating that **1** scaffold is not suited  
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7 for exploring the noncanonical pocket observed by Bista et al.<sup>26</sup> Interestingly, in this  
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9 particular group of compounds the drop of affinity towards MDM2 was not associated with  
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11 significant change in potency against MDMX, again demonstrating differences in the p53  
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13 binding pockets of those two proteins.  
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17 In parallel we evaluated the affinity of ethyl ester precursors (SI Table S2) of the **20-24**  
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19 series, but these compounds (**20a-24a**) have entirely lost their affinity both towards MDM2  
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21 and MDMX. This is relatively surprising given that the R<sub>2</sub> position is modified with much  
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23 larger substituents in **13-19**, but this phenomenon was not further evaluated in systematic  
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25 manner.  
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29 **Derivatives of Compound 1 Induce p53-target Genes and Arrest the Cell Cycle.** The  
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31 on target effect of our 1,4,5-trisubstituted imidazoles in a biological context of a living cell  
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33 was evaluated by assessing the expression level of p53, MDM2 and p21 proteins, which are  
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35 the products of well-established p53 target genes. All compounds that had K<sub>i</sub> values in the FP  
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37 assay lower than 2 μM were tested. The second generation MDM2 antagonist **RG7388**  
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39 (Roche; 4-[(2R,3S,4R,5S)-3-(3-Chloro-2-fluoro-phenyl)-4-(4-chloro-2-fluorophenyl)-4-  
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41 cyano-5-(2,2-dimethyl-propyl)-pyrrolidine-2-carbonyl]-amino}-3-methoxybenzoic acid,  
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43 named thereafter **2.1**)<sup>34</sup> was used as a positive control. **2.1** induced expression of all of the  
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45 three proteins already 7 h after the treatment (Figures 2A,B). The densitometry analysis  
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47 revealed that the level of p21 expression was increased even stronger after 24 h of the  
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49 treatment (Figure 2C). Out of the tested compounds, **1**, **13**, **14**, **15**, **16**, and **19** induced  
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51 accumulations of p53, MDM2 and p21, suggesting the activation of the p53 pathway (Figures  
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53 2A,B, SI Figure S1). From these compounds, only for **13**, **14** and **19** the induction of p21  
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3 expression was further increased at 24 h of the treatment as observed for **2.1**, possibly  
4 reflecting improved stability, retention or activity of the compounds in the cells (Figure 2C).  
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8 In parallel, the off target effects/general toxicity were assessed by comparing the influence  
9 of tested agents on the viability of p53<sup>wt</sup> U-2 OS and p53-null Saos-2 cells. Accordingly, the  
10 compounds most active in the western blot analysis induced growth inhibition of U-2 OS  
11 (p53<sup>wt</sup>) cells at lower concentrations than the Saos-2 (p53<sup>-/-</sup>) cells suggesting on target effect  
12 (Table 2). Compound **15** revealed high toxicity towards Saos-2 cells and was removed from  
13 further analysis. As a control, nutlin-3 was used in the MTT assay, confirming its selectivity  
14 towards the p53<sup>wt</sup> cells (Table 2). The remaining tested compounds were disqualified due to  
15 their weak impact on the growth of the U-2 OS cell line (IC<sub>50</sub> values above 30 μM, e.g. **3**, **18**  
16 or **20**) or high toxicity towards the Saos-2 cells (selectivity lower than 1.5, e.g. **2**, **6** or **15**).  
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29 Accumulation of p21 upon activation of p53 is expected to trigger cell cycle arrest in the  
30 p53<sup>wt</sup> cells. The effect of the five most potent compounds on the cell cycle progression was  
31 tested in U-2 OS cells. Treatment with the 5 μM concentration of **13**, **14** or **19** resulted in a  
32 markedly decreased S-phase and increased G1 fraction (Figure 3). At higher concentrations  
33 (10 μM) **14** and **19** resulted in nearly complete depletion of the S-phase compartment, while  
34 for **1**, **16** and **13** the increase of the G1 fraction, in the expense of S phase, was clearly  
35 observed (Figure 3). The observed extent of cell cycle arrest was comparable to that obtained  
36 using 1 μM **2.1**, a compound currently undergoing clinical trials.  
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48 Clearly, the selected compounds show the potency in blocking the progression of the cell  
49 cycle in a p53-dependent manner, and addition of the 2-morpholinoethyl or, especially, 6-  
50 succinyloxyhexyl substituent at position R<sub>2</sub> is profitable for the biological activity of  
51 compound **1**. In conclusion, compound **19** presents the best biological properties, as  
52 demonstrated by strong and sustained activation of p53-dependent expression of p21, the  
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3 highest selectivity in the MTT assay and the strongest induction of cell cycle arrest of p53<sup>wt</sup>  
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5 cells.  
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### 8 **Crystal Structure of MDM2-19 Complex Reveals Compound Induced Dimerization.**

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10 In order to better understand the structural basis of the interactions of evaluated compounds at  
11 the binding pocket of MDM2, we determined the crystal structure of **19** in complex with the  
12 p53 binding domain of MDM2(18-125) (construct of MDM2 comprising residues 18-125). **19**  
13 was selected as the most efficient, cell-available, p53-activating derivative. At the same time  
14 the compound is potentially directly suitable for further conjugation with biological carriers  
15 for its reactive group at a long aliphatic linker.  
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24 The crystals diffracted to the 1.85 Å resolution and contained four protein molecules in the  
25 asymmetric unit. Each protein contained a single inhibitor molecule, all of which were well  
26 defined by their respective electron densities (SI Figure S2). No additional inhibitor molecules  
27 were found within the structure. In each case the inhibitor locates at the p53 binding pocket  
28 and occupies all the subpockets (Phe19, Trp23 and Leu26) of a canonical three-finger  
29 pharmacophore (Figure 4). The interaction of **19** with MDM2 is defined primarily by  
30 hydrophobic contacts. The 6-chloroindole substituent inserts into tryptophan pocket and  
31 assumes an orientation almost identical to the indole side chain in the native p53-MDM2  
32 complex while the chlorine atom potentiates the interaction at the bottom of the binding  
33 pocket. Further comparable to p53 interaction, the nitrogen within 6-chloroindole participates  
34 in a hydrogen bond with the carbonyl oxygen of Leu54. As such, the overall arrangement of  
35 6-chloroindole moiety follows a classical binding observed in a large number of MDM2  
36 inhibitors of different classes and repeating the indole interaction involved in p53 binding.<sup>16</sup>  
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38 4-chlorobenzyl substituent fills the Leu26 pocket in an orientation in which the chlorine atom  
39 points towards Tyr100. Phe19 pocket accommodates 4-fluorophenyl ring directed at Tyr67.  
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60 The imidazole scaffold of **19** further contributes to the interaction by forming hydrophobic

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3 contacts with Val93. As such, the binding of the core of the compound and aromatic  
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5 substituents is almost identical to that previously described for **1.1**. This is not surprising since  
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8 **1.1** is a precursor of **19** (SI Figure S3).

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10 Most interesting are the interactions of the aliphatic linker unique to **19**. Comparable  
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12 affinities of **1** and **19** did not portend any pronounced protein – linker interactions. In fact, in  
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14 the crystal structure the linker protrudes outside the binding pocket. Only the disposition of  
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16 Gln72 and His73 sidechains differs from that observed in **1.1** containing structure. This  
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18 adjustment is necessary to accommodate the linker (SI Figure S3). Remarkably, however, the  
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20 protruding linker immediately contacts a second inhibitor molecule located within the  
21  
22 adjacent MDM2 molecule. The interaction is reciprocal as the linker of the inhibitor molecule  
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24 within the pocket of the adjacent MDM2 in turn contacts the inhibitor molecule within the  
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26 first MDM2. As such, the two MDM2 molecules form a dimer build around a dimer of two  
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28 inhibitor molecules (Figures 5A,B). The primary contacts between the inhibitor molecules  
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30 involve hydrogen bonds between the imidazole ring nitrogens and the nitrogens within the  
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32 aliphatic linker (Figure 5C). The overall dimer is further stabilized by reciprocal intra-MDM2  
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34 hydrogen bonds formed by Gln59 and Thr63 (Figure 5D). Moreover, the oxygen from the  
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36 carboxylic group terminating the aliphatic linker of each **19** molecule forms a water-mediated  
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38 contact with the side chains (Gln72, His96) of MDM2 (Figures 5A,C).

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41 To test if the observed **19**-mediated MDM2(18-125) dimerization was unrelated to the lack  
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43 of the N-terminal part of the MDM2 domain in the evaluated construct we have solved the  
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45 crystal structure of **19** in complex with a different construct of MDM2(1-125). Moreover,  
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47 these crystals were obtained in different conditions and belonged to a different space group  
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49 providing a partial control of the influence of solvent conditions and crystal packing. Despite  
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51 these differences the dimerization mode within both structures is virtually identical,  
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53 suggesting that dimer formation is compound induced rather than an effect of particular  
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3 experimental conditions. The MDM2 molecules within the dimers seen in the two crystal  
4 forms align with RMSD of 0.198 Å for corresponding C<sub>α</sub> atoms. The inhibitor dimers and  
5 interactions between the MDM2 molecules are also identical in both structures. The only  
6 difference is in amino acids 11-17 which were not present in the first construct, but are  
7 present and defined by electron density in the second structure. This fragment closes the  
8 binding pocket from both sides of the dimer (SI Figure S4), but has no significant effect on  
9 the mode of dimerization. Residues 1-10 were not defined by electron density.

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20 Small molecule induced dimerization of MDM2 has already been previously reported by  
21 Graves et al. and by Surmiak et al., respectively for **RO-2443** (Roche; (Z)-5-((6-chloro-7-  
22 methyl-1H-indol-3-yl)methylene)-3-(3,4-difluorobenzyl)imidazolidine-2,4-dione, named  
23 thereafter **3.1**)<sup>35</sup> and 3-pyrrolin-2-ones/2-furanones.<sup>36</sup> In all instances the dimer is mediated by  
24 direct interaction of the inhibitor moieties facing outside of the binding pocket of MDM2.  
25 However, when the structures of **19**, **3.1** and 3-pyrrolin-2-ones/2-furanones induced dimers  
26 are compared by aligning one of the MDM2 molecules within the dimer, the positions of the  
27 partner molecules do not significantly overlap demonstrating different overall dimer  
28 arrangement (SI Figures S5-S6). The interactions between the inhibitor molecules are also  
29 significantly different. In **19**, it is the amide within the linker of one inhibitor molecule that  
30 forms a hydrogen bond with the nitrogen of the imidazole ring of adjacent inhibitor and *vice*  
31 *versa* (Figure 5). In **3.1**, aromatic stacking between the indolyl-hydantoin groups of adjacent  
32 inhibitors is primarily involved (SI Figure S5), whereas in 3-pyrrolin-2-ones/2-furanones it is  
33 a water mediated interaction between the inhibitor molecules and a halogen bond formed by  
34 the inhibitor and adjacent protein molecule (SI Figure S6).

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55 Overall, although target protein dimerization through the interaction of exposed moieties of  
56 inhibitors is common for **19**, **3.1** and 3-pyrrolin-2-ones/2-furanones, the detailed molecular  
57 mechanisms and the overall geometry of the resulting dimers are largely dissimilar.  
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### **19 Induces Dimerization of MDM2 in Solution and Dissociates p53-MDM2 Complex.**

The arrangement of the molecules in the crystal does not necessarily have to reflect the real biological assembly. Therefore, we evaluated if dimerization of MDM2 is induced by **19** in solution. The retention time characterizing MDM2 in analytical gel filtration decreased in the presence of **19** indicating formation of higher molecular weight species, presumably dimers (based on column calibration with globular protein standard). **19**-induced shifts in the elution volume were observed for all MDM2 constructs evaluated in this study (1-118, 1-125, 18-125; Figure 6A; SI Figure S7), so the observed dimerization is not induced by artificially exposing certain MDM2 surface.

Increase in the molecular mass of functional assembly results in increased relaxation time in NMR manifesting in signal broadening in the recorded spectra. To confirm **19**-induced dimerization of MDM2, the protein was titrated with the tested compound and the line width of well separated aliphatic signals in the region from -0.5 ppm to 1.0 ppm of 1D <sup>1</sup>H NMR spectra was monitored. Line width changes were not observed upon titration with **2.1**, a reference compound known not to affect the monomeric state of MDM2 in solution. Titration with **19** resulted in marked broadening of peaks indicating the dimerization (Figure 6B, SI Figure S7). Similar results were obtained regardless the MDM2 construct used (1-118, 18-125 and 1-125). These results further support the **19**-induced MDM2 dimerization in solution.

Next we asked if **19** is able to dissociate an existing p53-MDM2 complex. To test this, we employed a binding competition experiment, known as the 1D AIDA-NMR (Antagonist-Induced Dissociation Assay).<sup>37</sup> The method relies on monitoring the line width changes associated with the changes in the molecular weight upon complex formation. The well separated, sharp signal originating from Trp23 of p53 transactivation domain broadens to the level of noise upon binding to MDM2. Displacement of p53 peptide from MDM2 complex by an MDM2 inhibitor results in the recovery of the specific signal in 1D <sup>1</sup>H NMR spectrum.

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3 Upon titration of p53-MDM2 complex with **19**, recovery of the specific Trp23 signal was  
4  
5 observed, demonstrating that the compound effectively dissociates the p53-MDM2 interaction  
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7 (Figure 6C), liberating p53 from the inhibition by its negative regulator.  
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## 17 CONCLUSIONS

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20 Derivatization of the previously reported **1.1**, first into compound **1** and later by modification  
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22 of the carboxyl moiety of the chloroindole scaffold, has led to a number of compounds with  
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24 affinities comparable to the starting structure, but characterized by significantly improved  
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26 activity in cell based assays. The best obtained compounds activated p53 in cancer cells to the  
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28 level comparable to the golden standard–nutlin-3a, that is induced the cell cycle arrest,  
29  
30 increased the expression of proapoptotic p21 and had a markedly increased cytotoxicity  
31  
32 towards p53 positive cells. Crystal structure of an example compound (**19**) revealed a  
33  
34 classical three finger pharmacophore binding mode identical to that of **1.1**, but at the same  
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36 time an unexpected, inhibitor induced dimerization of the target protein. The dimerization was  
37  
38 confirmed in solution. Such unusual binding mode provides entirely new repertoire of  
39  
40 optimization possibilities. Further, the long aliphatic linkers, containing functional groups,  
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42 allow for future attachment of presented series of compounds to biological carriers (e.g.  
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44 hyaluronic acid)<sup>38</sup> for improved pharmacodynamics and better delivery.  
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## 53 EXPERIMENTAL SECTION

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55 **Synthesis.** Compounds **1** and **20-24** were obtained in the 3CR van Leusen reaction. **1** was  
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57 further modified at position R<sub>2</sub> to obtain amide (**2-13**) and ester (**14-19**) derivatives using  
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59 classical chemistry. The purity of all final compounds - determined by the chromatographic  
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3 UPLC or elemental analysis - was 95% or higher. For details of synthesis and analysis, see  
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5 the SI.  
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8 **Protein Expression and Purification.** Variants of the N-terminal domain of human  
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10 MDM2 (1-118; 1-125 and 18-125) were cloned into the pET-20 (Novagen) and expressed in  
11  
12 *E. coli* strain BL21-CodonPlus(DE3)-RIL as described previously.<sup>39</sup> In brief, cells were  
13  
14 cultured at 37°C. Protein expression was induced with 1 mM IPTG at OD<sub>600</sub> of 0.8 and  
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16 cultured for additional 5 h at 37°C. Cells were collected by centrifugation and lysed by  
17  
18 sonication. Inclusion bodies were collected by centrifugation, washed with PBS containing  
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20 0.05% Triton-X100 and subsequently solubilized in 6 M guanidine hydrochloride in 100 mM  
21  
22 Tris-HCl, pH 8.0, containing 1 mM EDTA and 10 mM β-mercaptoethanol. The protein was  
23  
24 dialyzed against 4 M guanidine hydrochloride pH 3.5 supplemented with 10 mM β-  
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26 mercaptoethanol. Following, the protein was refolded by dropwise addition into 10 mM Tris-  
27  
28 HCl, pH 7.0, containing 1 mM EDTA and 10 mM β-mercaptoethanol and slow mixing  
29  
30 overnight at 4°C. Ammonium sulfate was added to the final concentration of 1.5 M and the  
31  
32 refolded protein was recovered on Butyl Sepharose 4 Fast Flow (GE Healthcare). The protein  
33  
34 was eluted using 100 mM Tris-HCl pH 7.2 containing 5 mM β-mercaptoethanol and further  
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36 purified by gel filtration on HiLoad 16/600 S75 (GE Healthcare) in 5 mM Tris-HCl pH 8.0  
37  
38 containing 50 mM NaCl and 5 mM β-mercaptoethanol (crystallization buffer) or in 50 mM  
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40 phosphate buffer pH 7.4 containing 150 mM NaCl and 5 mM DTT (FP/NMR buffer).  
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48 N-terminal domain of human MDMX (1-134) was cloned into pET-46Ek/LIC vector  
49  
50 (Novagen). The vector was transformed into *E. coli* strain BL21-CodonPlus(DE3)-RIL cells.  
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52 The cells were cultured at 37°C and protein expression was induced with 0.5 mM IPTG at  
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54 OD<sub>600</sub> nm of 0.6. The recombinant protein expression was carried for 12 h at 20°C. The  
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56 protein was purified in native conditions by metal affinity chromatography. The preparation  
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3 was polished by gel filtration on HiLoad 16/600 S75 (GE Healthcare) in 50 mM phosphate  
4 buffer pH 7.4 containing 150 mM NaCl and 5 mM DTT (FP/NMR buffer).  
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8 **Fluorescence Polarization Assay.** The assay was performed in 50 mM NaCl, 10 mM Tris  
9 pH 8.0, 1 mM EDTA containing 5% DMSO. To determine the optimal concentration of the  
10 protein for the competition binding assay, the effective concentration of MDM2 (1-118) and  
11 MDMX (1-134) was each time ascertained by determining the apparent  $K_d$  towards 5'-FAM-  
12 LTFEHYWAQLTS (P2; 10 nM). Competition assay was performed by contacting serial  
13 dilutions of tested compounds with 10 nM P2 at protein concentration yielding  $f_0=0.8$ .<sup>40</sup>  
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Fluorescence polarization was determined at 485 nm excitation and 535 nm emission 15 min  
after mixing all assay components. All tests were performed using Corning black 96-well  
NBS assay plates at room temperature.

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**Cell Lines and Treatment with Compounds.** U-2 OS (p53<sup>wt</sup>), HCT 116 (p53<sup>wt</sup>) and  
Saos-2 (p53<sup>-/-</sup>) cells were cultured as monolayers in McCoy's 5A Medium containing L-  
glutamine (Lonza) and supplemented with 10% fetal bovine serum (Biowest) at 37°C and 5%  
CO<sub>2</sub> in a humidified atmosphere. All compounds were prepared as 50 mM stock solutions in  
DMSO. The cells were exposed to the inhibitors for indicated time periods. In each  
experiment, the final concentration of DMSO was kept constant and below detectable effect  
level.

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**Western Blot Analysis.** U-2 OS cells and HCT 116 cells (120 000 cells per well) were  
seeded on 12-well transparent plates (Falcon). The inhibitors were added 24 h following  
seeding and the cells were cultured for additional 7 or 24 h. Cells were lysed with RIPA  
buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma). The protein lysate was  
clarified by centrifugation and analyzed on 12% SDS polyacrylamide gel (Bio-Rad). The  
proteins were transferred to PVDF membrane (Merck Millipore). The membranes were  
blocked for 1 h in 5% BSA (Bioshop) in TTBS (100 mM Tris-HCl pH 8.0 containing 0.05%

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3 Tween-20 and 150 mM NaCl). Blots were probed with following antibodies: anti-MDM2  
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5 (Thermo Fisher Scientific, cat. no. 700555), p21 (Cell Signaling, cat. no. 2947), both at  
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7 1:2000 dilution, anti-p53 (Santa Cruz Biotechnology, cat. no. sc-6243) at 1:200 dilution or  
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9 anti-GAPDH (Cell Signaling, cat. no. 2118) at 1:3000 dilution. Anti-rabbit antibody  
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11 conjugated with horseradish peroxidase (Cell Signaling, cat. no. 7074) at 1:3000 dilution was  
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13 used to detect the primary antibodies. The blots were developed using ECL Western Blotting  
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15 KIT (Bio-Rad) and visualized using ChemiDoc System (Bio-Rad). Densitometric analysis  
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17 was performed using ImgeLab software (Bio-Rad).  
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22 **Cell Viability Assay.** U-2 OS cells (500 cells per well) and Saos-2 cells (1500 cells per  
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24 well) were seeded on 96-well transparent plates (Falcon). Cells were treated with inhibitors  
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26 24 h following the seeding and cultured for additional 5 days. Thiazolyl blue tetrazolium  
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28 bromide (MTT) was added at the final concentration of 500 ng mL<sup>-1</sup> and the cells were  
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30 incubated for 1 h at 37°C. The medium was removed and formazan crystals were dissolved in  
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32 isopropanol containing 40 mM HCl. Absorbance was determined at 570 nm relative to  
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34 650 nm reference. Data was normalized to DMSO-treated control. Data was analyzed using  
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36 Origin software (OriginLab). IC<sub>50</sub> values were calculated by fitting of 'Dose Resp' curves to  
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38 experimental datasets.  
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43 **Cell Cycle Analysis.** U-2 OS cells were seeded at 300 000 per 60·15 mm cell culture dish  
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45 (Eppendorf), treated with tested inhibitors for 24 h, trypsinized and fixed with 70% ethanol.  
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47 The cell concentration was adjusted to 1 x 10<sup>6</sup> cells/sample and DNA was labeled with PI-  
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49 RNase solution (PBS, 5 µg mL<sup>-1</sup> propidium iodide, 0.01 mg mL<sup>-1</sup> RNase A) for 30 min at  
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51 25°C. The cells analyzed for cell cycle DNA content using LSRFortessa cell analyzer (BD  
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53 Biosciences).  
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58 **Crystal Structure Determination.** Human MDM2 (residues 18-125 and 1-125) was  
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60 prepared in 5 mM Tris-HCl pH 8.0 containing 50 mM NaCl and 5 mM β-mercaptoethanol.

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3 Molar excess (3×) of **19** was added and the complex was concentrated to 20 mg mL<sup>-1</sup>.  
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5 Screening for crystallization conditions was performed using sitting drop vapor diffusion  
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7 setup and commercially available buffer sets (Qiagen, Hampton Research). Crystals of the  
8  
9 MDM2(18-125)-**19** complex appeared after few days at 4°C in 0.1 M Na HEPES pH 7.5  
10  
11 containing 10% isopropanol and 20% PEG 4000. The MDM2(1-125)-**19** complex crystallized  
12  
13 at 4°C in 0.1 M HEPES pH 7.5 containing 0.2 M NaCl and 25% PEG 3350. The data  
14  
15 collection and refinement description and statistics (SI Table S6) are summarized in the SI.  
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19  
20 **Analytical Gel Filtration.** MDM2(18-125, 1-118 and 1-125) at 1 mg mL<sup>-1</sup> was contacted  
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22 with 3× molar excess of tested inhibitor for 30 min and the distribution on different molecular  
23  
24 weight species was analyzed by gel filtration in the crystallization buffer using S75 10/300  
25  
26 GL column (GE Healthcare).  
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30 **NMR Experiments.** All NMR spectra were acquired at 300 K using a Bruker Avance 600  
31  
32 MHz spectrometer. Uniform <sup>15</sup>N isotope labelling was achieved by expression of the protein  
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34 in the M9 minimal medium containing <sup>15</sup>NH<sub>4</sub>Cl as the sole nitrogen source. MDM2(1-  
35  
36 118)<sup>41,42</sup> was prepared in 50 mM phosphate buffer pH 7.4 containing 150 mM NaCl and  
37  
38 5 mM DTT. 10% (v/v) of D<sub>2</sub>O was added to the samples to provide lock signal. Water  
39  
40 suppression was carried out using the WATERGATE sequence.<sup>43</sup> Stock solutions of  
41  
42 inhibitors used for titration were prepared in d<sub>6</sub>-DMSO. The spectra were processed with  
43  
44 TopSpin 3.2 software. <sup>1</sup>H-<sup>15</sup>N heteronuclear correlations were obtained using the fast HSQC  
45  
46 pulse sequence.<sup>44</sup> Assignment of the amide groups of MDM2 was obtained after <sup>45</sup>.  
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49 The AIDA experiments were performed according to <sup>37</sup>.  
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## 55 ASSOCIATED CONTENT

### 56 Supporting Information

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3 Details on chemical synthesis of compounds, X-ray, FP and NMR may be found in the  
4  
5 Supporting Information. This material as well as Molecular Formula Strings are available free  
6  
7 of charge via the Internet at <http://pubs.acs.org>.  
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### 10 Accession Codes

11  
12 The final models and structure factors for MDM2-**19** complexes were deposited into Protein  
13  
14 Data Bank with the following accession numbers: 5J7G (MDM2 18-125) and 5J7F (MDM2  
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16 1-125).  
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#### 31 Author Contributions

32  
33  
34 # These authors contributed equally to this work.  
35

#### 36 Notes

37  
38 The authors declare no competing financial interest.  
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#### 34 **ABBREVIATIONS USED**

35  
36  
37 3CR, 3-Component Reaction; AIDA, Antagonist-Induced Dissociation Assay; FP,  
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39 Fluorescence Polarization; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; MTT, 3-  
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41 (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MDM2, Murine Double  
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43 Minute-2; MDMX, Murine Double Minute-X; PVDF, Polyvinylidene Fluoride; RMSD, Root  
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45 Mean Square Deviation.  
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11 **TABLES, FIGURES AND FIGURE LEGENDS**  
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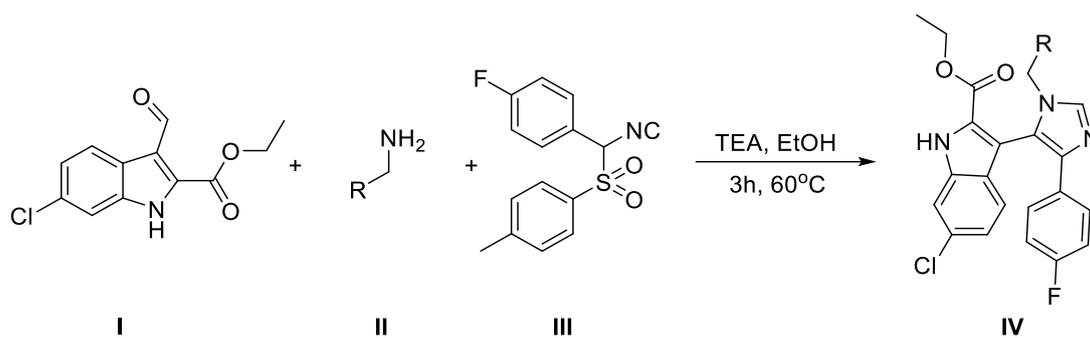
**Table 1.** Inhibitory activities of compound **1** and its derivatives against the p53-MDM2/MDMX interaction using the FP assay.

Code	R <sub>2</sub>	K <sub>i</sub> M2 [μM]	K <sub>i</sub> MX [μM]	Code	R <sub>2</sub>	K <sub>i</sub> M2 [μM]	K <sub>i</sub> MX [μM]
<b>1</b> *		0.006	3.15	<b>13</b>		0.121	n.a.#
<b>2</b>		0.225	n.a.	<b>14</b>		0.017	40.2
<b>3</b>		0.023	14.7	<b>15</b>		0.059	n.a.
<b>4</b>		0.038	29.6	<b>16</b>		0.015	9.75
<b>5</b>		2.31	n.a.	<b>17</b>		0.098	n.a.
<b>6</b>		0.038	n.a.	<b>18</b>		0.054	12.4
<b>7</b>		28.0	n.a.	<b>19</b>		0.058	26.3
<b>8</b>		0.008	35.3	<b>20a</b> <sup>±</sup>		6.02	n.a.
<b>9</b>		0.656	27.4	<b>20</b> <sup>°</sup>		0.021	4.91
<b>10</b>		n.a.	n.a.	<b>21a</b>		n.a.	n.a.
<b>11</b>		n.a.	n.a.	<b>21</b>		0.060	4.40
<b>12</b>		n.a.	n.a.	<b>22a</b>		n.a.	n.a.
				<b>22</b>		0.433	4.71
				<b>23a</b>		n.a.	n.a.
				<b>23</b>		0.322	6.76
				<b>24a</b>		n.a.	n.a.
				<b>24</b>		0.157	4.17

\*compounds **1-19** have 4-chlorophenyl group in R<sub>1</sub> position; °compounds **20-24** have hydroxyl group in R<sub>2</sub> position; <sup>±</sup>**a** stands for ethyl ester (**20a-24a**) of the respective acidic forms of **20-24**; #n.a. stands for not active.

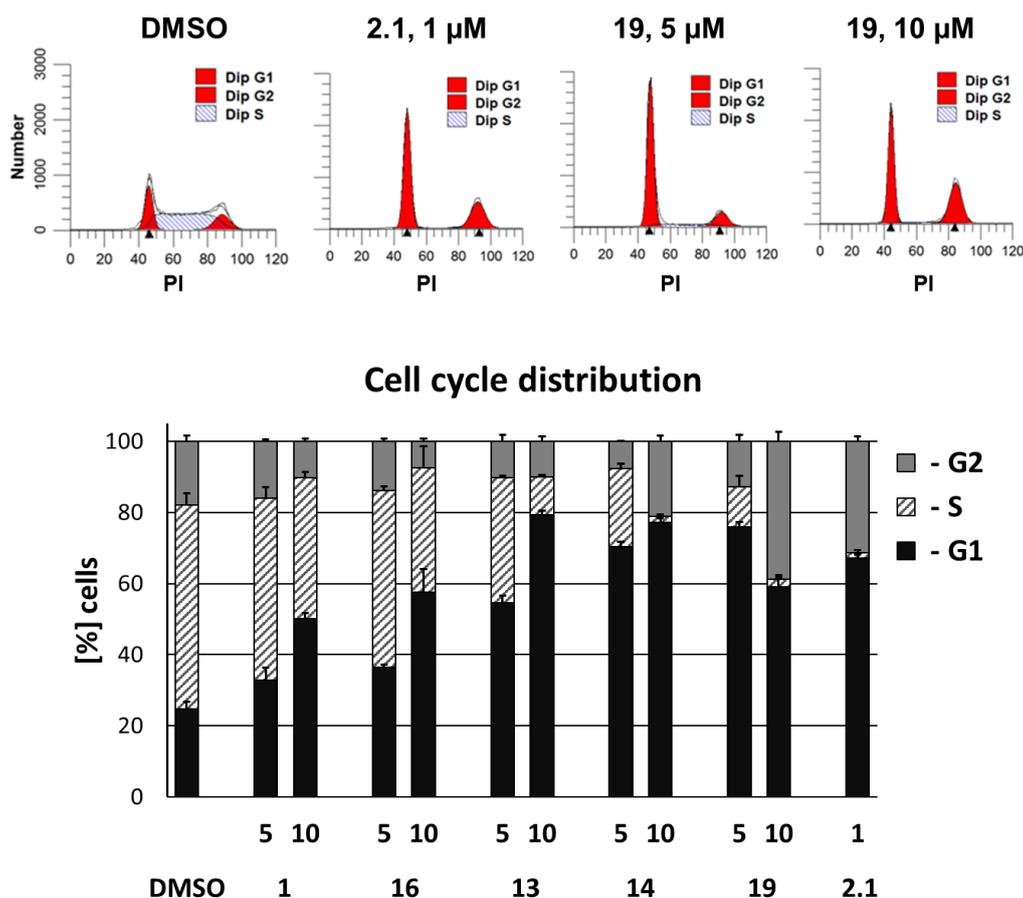
**Table 2.** Selectivity of the tested compounds towards the p53 positive cells. IC<sub>50</sub> values were obtained from the MTT assay. Selectivity is calculated as a ratio of IC<sub>50</sub> values towards the p53 negative (Saos-2) and positive (U-2 OS) cells.

Code	U-2 OS IC <sub>50</sub> [μM]	Saos-2 IC <sub>50</sub> [μM]	Selectivity
<b>19</b>	11.08 ± 1.83	38.56 ± 3.61	3.48
<b>1</b>	17.42 ± 3.49	55.82 ± 13.49	3.20
<b>16</b>	5.71 ± 1.39	12.73 ± 2.66	2.23
<b>13</b>	3.67 ± 0.28	7.15 ± 1.33	1.95
<b>14</b>	7.27 ± 0.63	11.69 ± 1.30	1.61
<b>Nutlin-3</b>	2.66 ± 0.39	20.16 ± 4.71	7.59

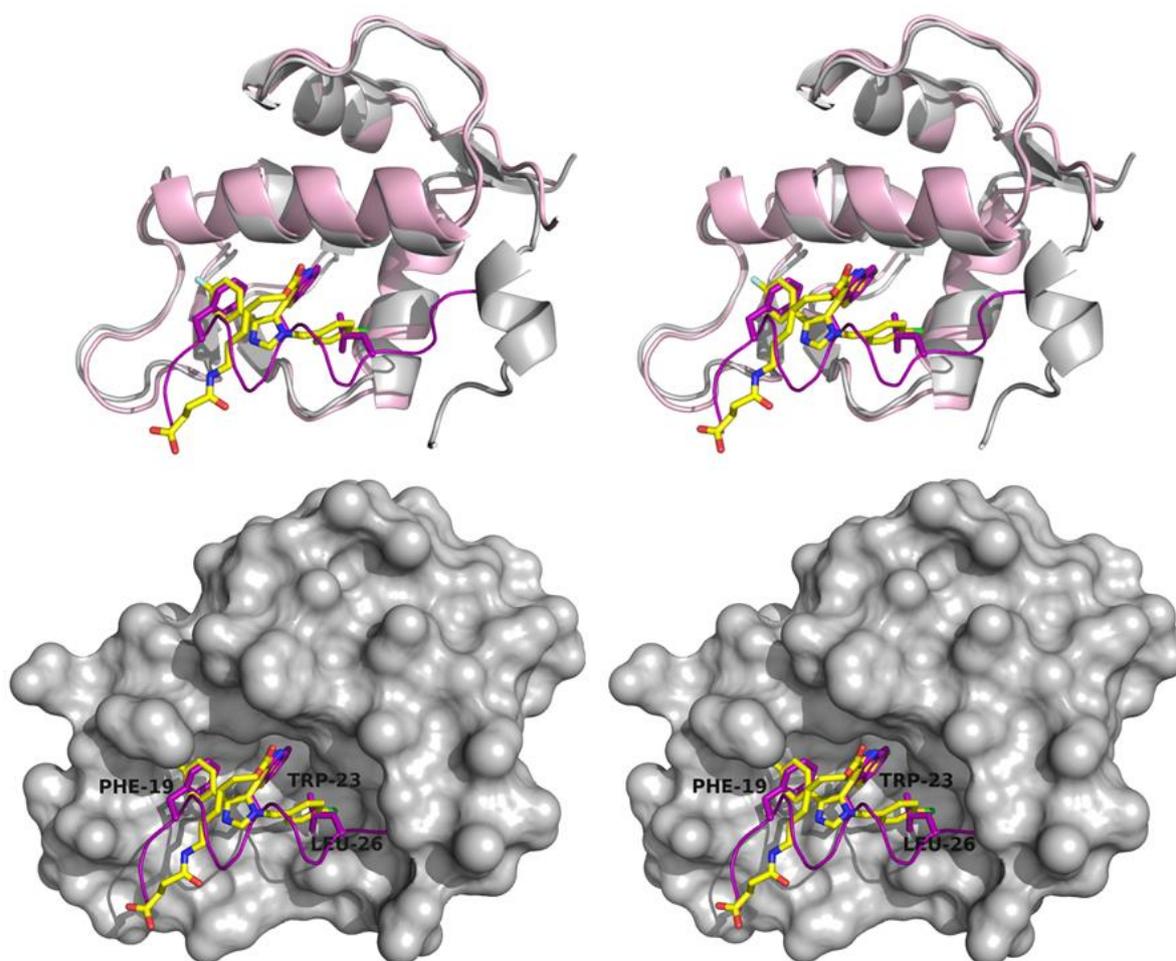


**Figure 1.** The Van Leusen reaction used for the synthesis of the series scaffold. The substituents are varied by changing the amine and isocyanide components (for details see the SI).

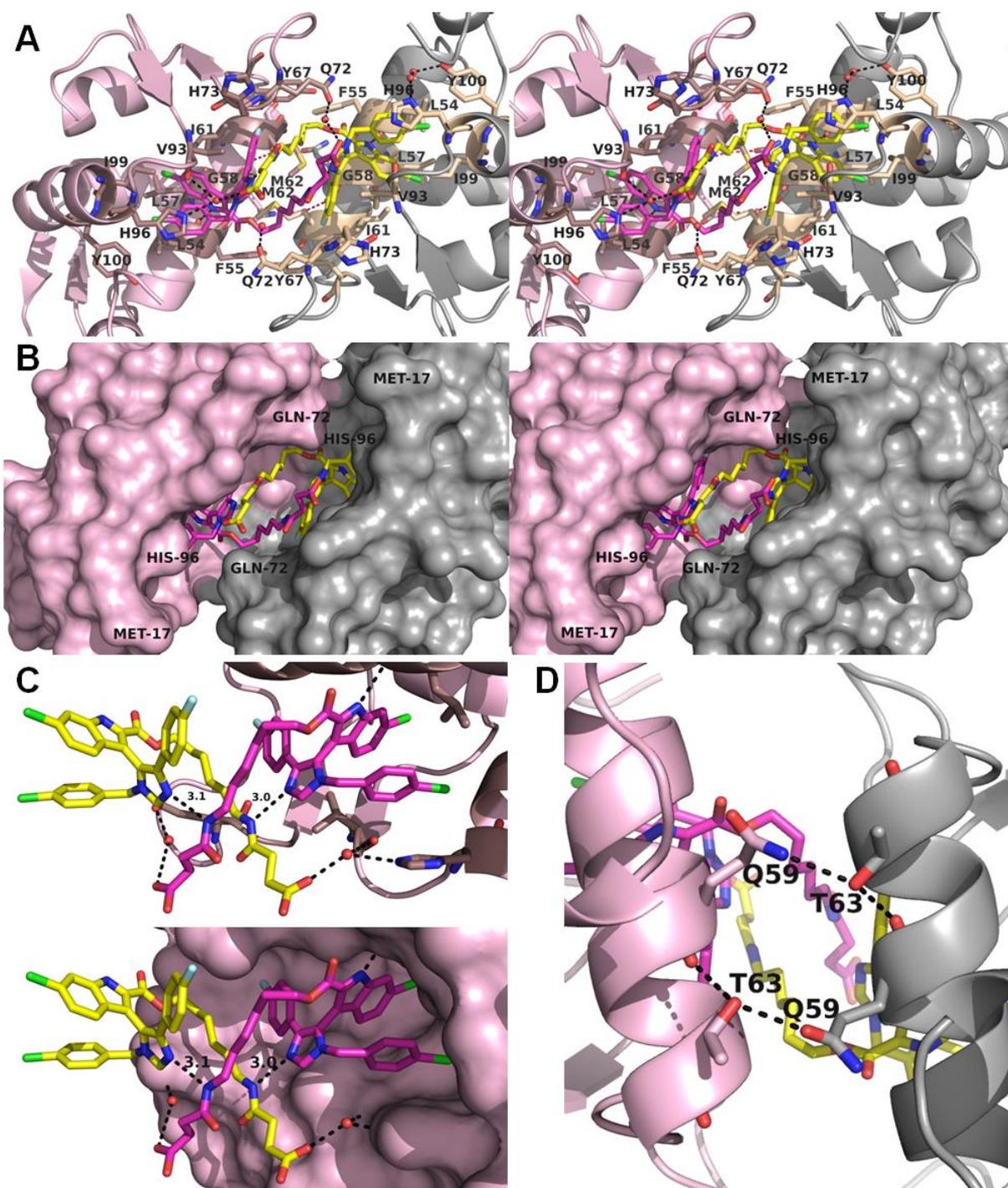




**Figure 3.** Compound **19** induces cell cycle arrest in the U-2 OS (p53<sup>+/+</sup>) cells. The cells were treated with DMSO, 5 or 10 μM of the tested compounds, or with 1 μM of **2.1** (positive control) for 24 h. The cells were fixed and stained with propidium iodide, followed by flow cytometry analysis of DNA content. Upper panel: exemplary histograms. Lower panel: Cell cycle distribution shown as the mean ± SD of two independent experiments.

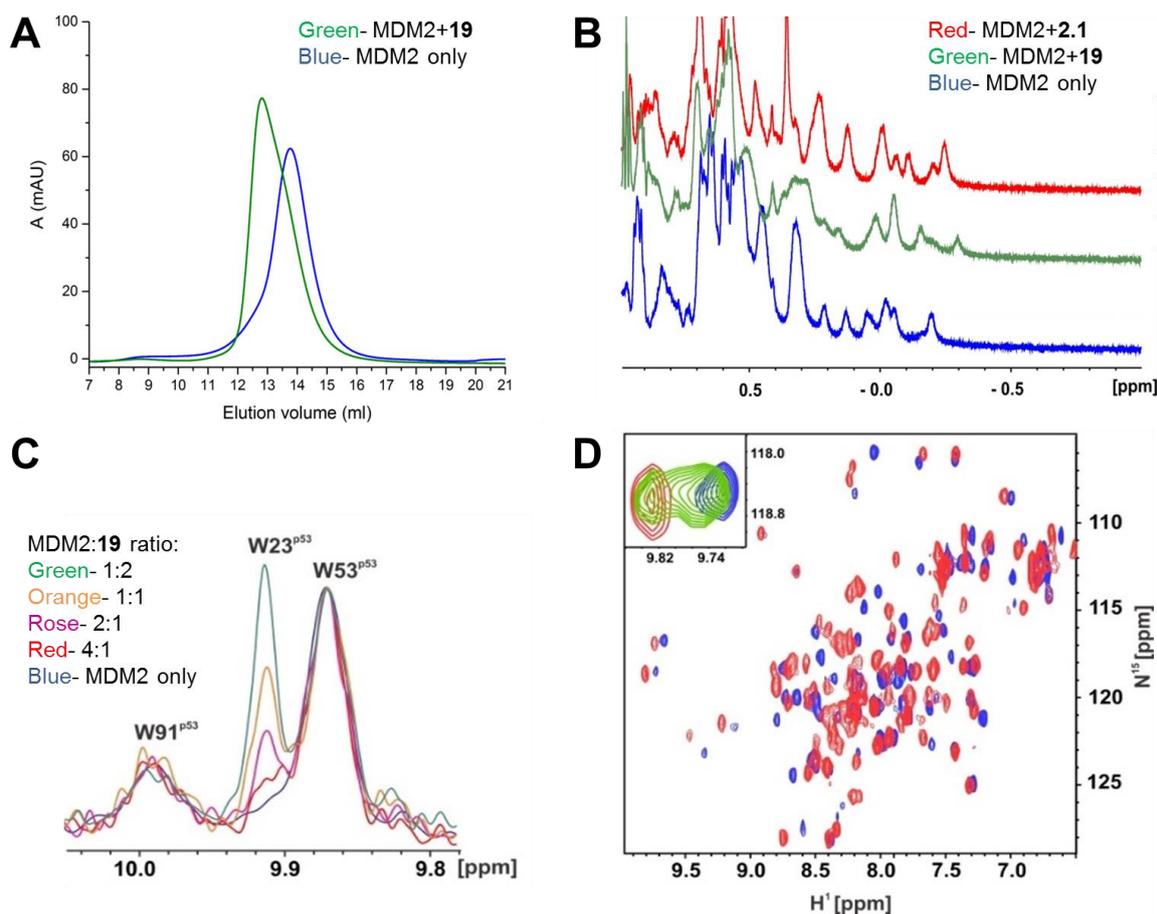


**Figure 4.** Interaction of MDM2 (residues 18-125) with **19** in comparison with the MDM2-p53 peptide complex (PDB ID: 1YCR). Stereoviews in cartoon (upper panel) or surface representations (lower panel) are shown. MDM2 from the MDM2-**19** complex is shown in grey, whereas from the MDM2-p53 complex – in pink. **19** is shown as yellow sticks, the p53 peptide – as a violet ribbon with the three key interacting amino acids depicted as sticks.



**Figure 5.** Crystal structure reveals the **19**-induced dimerization of MDM2. A. Stereoview of the structure of the MDM2(18-125)-**19** complex. MDM2 chains A (pink) and B (grey) are shown in the cartoon representation. Inhibitors are shown as magenta and yellow sticks. Residues involved in the interactions with **19** are shown as sticks in salmon (chain A) or wheat (chain B) and labeled. Hydrogen bonds contributed by the inhibitors are depicted as black dashes. B. Stereoview of the MDM2(18-125)-**19** complex – surface representation.

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3 Orientation and color coding same as in panel A. C. Inter-inhibitor interactions – the close-up  
4 view of the MDM2 binding pocket shown as a pink cartoon (upper panel) or surface (lower  
5 panel) with the buried inhibitor molecule and adjacent inhibitor molecule. The second MDM2  
6 molecule within the dimer is not shown. Hydrogen bonds are highlighted. D. Direct protein-  
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8 protein intermonomer interactions are formed between  $\alpha$ -helices of the adjacent MDM2  
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10 molecules. The interacting residues are shown as sticks; hydrogen bonds are highlighted in  
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**Figure 6.** Compound **19** induces dimerization of MDM2 in solution and dissociates the MDM2-p53 complex. A. Increased retention time of MDM2 in analytical gel filtration in the presence of **19** indicates formation of the dimer. B. The aliphatic region of  $^1\text{H}$  NMR spectra of MDM2(18-125) in the 1:1 complex with indicated compounds. **2.1** binds MDM2, but does not induce dimer formation. Broadening of NMR resonance peaks in the MDM2-**19** complex indicates the MDM2 dimerization. C. 1D AIDA NMR. The selected region of the 1D NMR spectrum is shown, containing resonances originating from W23, W53 and W91 of p53. The W23 peak is not present in the spectrum of the p53-MDM2 complex, but is recovered after addition of **19**, indicating compound induced dissociation of the complex. D. The HSQC spectrum of the  $^{15}\text{N}$ -labeled MDM2 (blue) superimposed with the spectrum after addition of **16** in the 1:1 molar ratio (red). The inset shows an intermediate state of a selected peak at the MDM2:**16** molar ratio equal to 2:1 (green). NMR signal splitting indicates strong interaction at  $K_D$  below  $1\ \mu\text{M}$ .

## TABLE OF CONTENTS GRAPHIC

