Regular Article

Synthesis and Optimization of New 3,6-Disubstitutedindole Derivatives and Their Evaluation as Anticancer Agents Targeting the MDM2/MDM*x* Complex

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Twelve derivatives of the general formula 3-substituted-6-chloroindoles were synthesized and tested for their growth inhibitory effects *versus* p53$^{+/+}$ colorectal cancer HCT116 and its p53 knockout isogenic cells; colorectal cancer cell p53$^{-/-}$ SW480; the lung cancer cell line p53$^{-/-}$ H1299; mouse embryonic fibroblasts (MEF) p53$^{+/+}$ and its p53 knockout isogenic cells. The compounds were also evaluated for their ability to induce p53 nuclear translocation and binding to murine double minute 2 (MDM2) and murine double minute 4 (MDM4). Of these, compound 5a was the most active in inhibiting the growth of cells, with selectivity towards the p53$^{+/+}$ cell lines, and it showed stronger binding to MDM4 rather than MDM2. The activity profile of compound 5a is strongly similar to that of Nutlin-3.

Key words indole derivative; p53; murine double minute 4 (MDM4); murine double minute 2 (MDM2)

In nearly all human cancers inactivation of tumor suppressor p53 can be among the underlying causes of the uncontrolled cell proliferation, that is why the reactivation of the p53 protein is considered one of the attractive approaches for cancer treatment that is currently targeted.[1)]

p53 plays a crucial role in the prevention of cancer development through acting as "the guardian of the genome" by activation of the transcription of a number of genes that are induced in response to various stress signals and are responsible for the regulation of the cell cycle check points, DNA repair, and finally induction of apoptosis.[2–5)]

Two major structurally related oncoproteins: murine double minute 2 (MDM2) and 4 (MDM4/*x*) regulate the activity of p53 through three parallel mechanisms: (1) MDM2/*x* binds to the transactivation domain (TAD) of p53 resulting in the inhibition of p53-responsive gene expression, (2) MDM2/*x* exports p53 from the nucleus where it can no longer activate transcription, and (3) by ubiquiltylating the MDM2–p53 complex causing it to undergo proteosomal degradation.[6–8)] MDM*x* is clearly involved in the first two mechanisms through formation of heterodimers with MDM2.[9)]

In approximately half of known cancer cases, p53 activity has been nullified by mutations and other genomic alterations in the DNA-binding domain of p53.[1)] In most of remaining cancer cases, the p53 retains its wild-type form, but it becomes functionally inactive by the effect of the overexpressed MDM2/*x* oncoprotein.[6)] Non-peptide small-molecules which inhibit the MDM2/*x*–p53 protein–protein interaction (PPI) can restore the normal activity of the wild-type p53 in MDM2/*x*-overexpressing cells and lead to selective apoptosis or cell cycle arrest in cancer cells.[10–14)]

X-Ray crystallography has established the structural basis of p53–MDM2 interaction. The crystal structure showed that the interaction occurred between a small but deep hydrophobic cleft in the MDM2 and a short α helix formed by residues 13–29 of p53 in which the three hydrophobic amino acids (Phe19, Trp23, Leu26) play a crucial role.[15)] Based on these interactions various classes of small-molecule MDM2 inhibitors have been reported. Nutlins, *cis*-imidazoline analogues, are the prototypes of this class of inhibitor.[10,16,17)] Subsequently, several spiro-oxindole derivatives *e.g.* MI-219 and 1,4-benzodiazepine-2,5-diones *e.g.* TDP222669, were introduced (Fig. 1). More recently, indolyl-hydantoin derivatives (*e.g.* RO-2443) were reported to potently block p53 binding with both MDM2 and MDM*x* by inhibitor-driven homo- and/or heterodimerization of MDM2 and MDM*x* proteins.[18,19)]

In this report, we explore the scope and limitations of indolylhydantoin compounds by synthesizing twelve novel small organic in the context of compound RO-2443 reported by Graves *et al.*[19)] The new molecules possess a 6-chloroindole ring, the core hydantoin ring is kept or changed to the structurally related thiohydantoin or thiazolidinedione. The terminal nitrogen was substituted by different halogen-substituted benzyl derivatives or 3,4-difluorobenzoyl substituent. The compounds were tested for their effects on cell lines and models with different expression state to p53 and for p53 nuclear translocation as shown hereunder.

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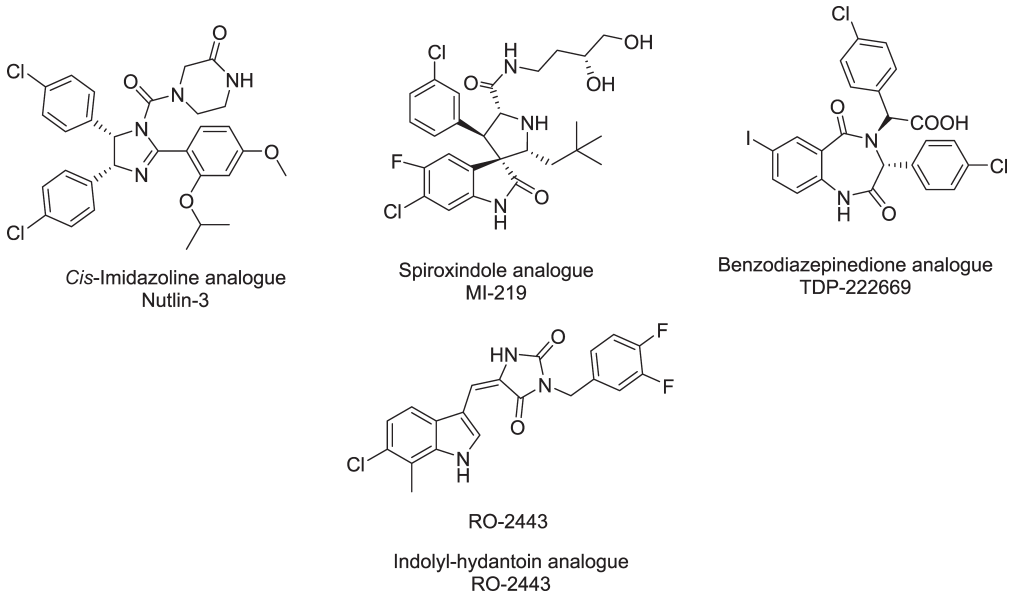
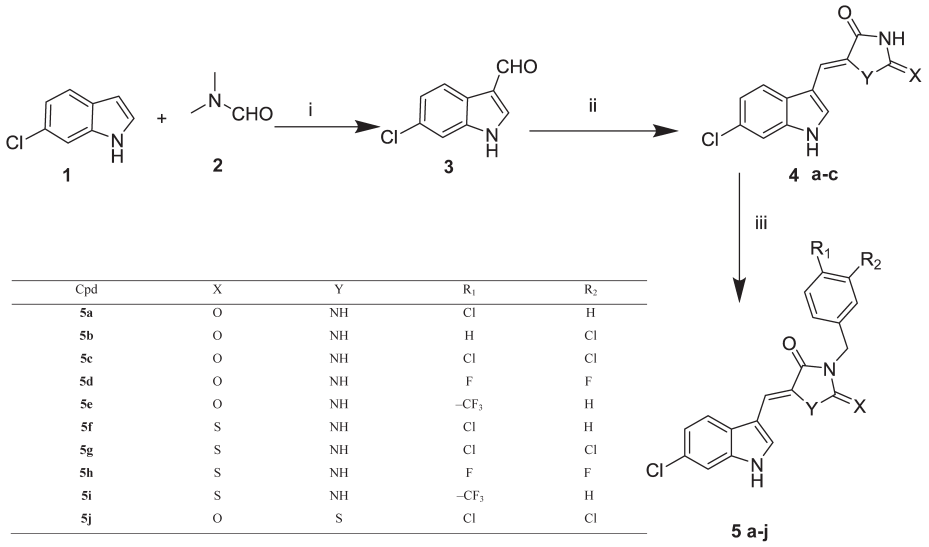


Fig. 1. Representative Examples of Known MDM2 and MDM4 Antagonists



Cpd	X	Y	R_1	R_2
5a	O	NH	Cl	H
5b	O	NH	H	Cl
5c	O	NH	Cl	Cl
5d	O	NH	F	F
5e	O	NH	$-CF_3$	H
5f	S	NH	Cl	H
5g	S	NH	Cl	Cl
5h	S	NH	F	F
5i	S	NH	$-CF_3$	H
5j	O	S	Cl	Cl

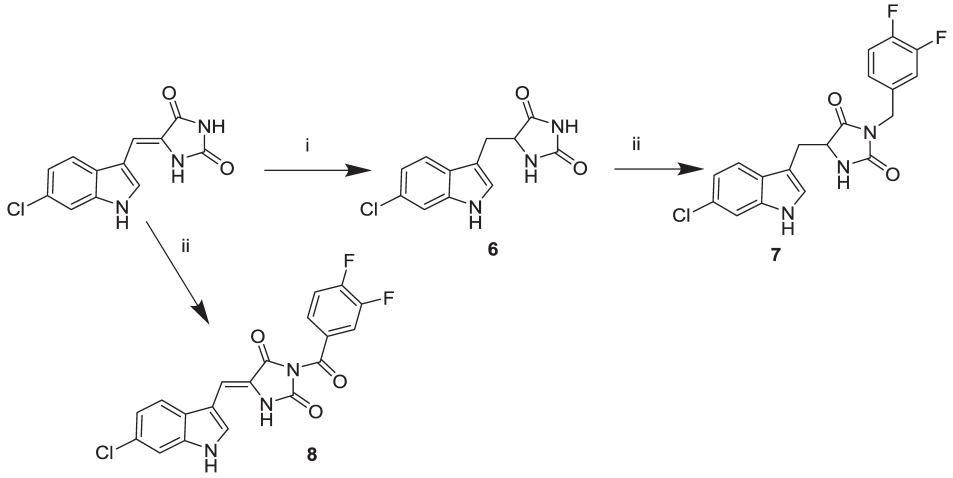
i) $POCl_3$, s: DMF, 1.5 h, 0°C; 2 h, room temp. ii) Hydantoin (**4a**) or thiohydantoin (**4b**) or thiazolidinedione (**4c**), $NaHCO_3$, s: H_2O or ethanol, 70°C, pH=7; $HOCH_2CH_2NH_2$, 90°C; overnight, reflux. iii) Substituted benzyl chlorides, K_2CO_3, C: KI, s: DMF, 45 min, reflux.

Chart 1

Results and Discussion

The synthetic protocol of the target compounds is depicted in Charts 1 and 2. In brief, the 6-chlorindole-3-carboxaldehyde was prepared by reaction of 6-chloroindole with dimethylformamide and phosphorus oxychloride, the arylidenes (**4a**–**c**) were formed by reaction of the aldehyde with the commercially available hydantoin, thiahydantoin or thazolidinedione, followed by arylation by the appropriate benzyl halide, Chart 1. To prepare the saturated, flexible analogue of **5d** (compound **7**), intermediate **4a** was reduced using excess sodium borohydride followed by benzylation using the 3,4-difluorobenzyl chloride. Also, compound **4a** was benzoylated to obtain the oxo-analogue of **5d** (compound **8**), Chart 2. All the synthesized compounds were sufficiently characterized by ^{1}H-, ^{13}C-NMR as well as LC-MS. The analytical data were consistent with the provided molecular structures of the designed compounds.

As a preliminary assay, all the compounds were tested for their potential growth inhibitory activity against the wild type ($p53^{+/+}$) HCT116 human colorectal carcinoma cell line. The resulting activities of the compounds are shown in Table 1. All the tested compounds showed anticancer activity against HCT116 with IC_{50} values ranging from 2.83 to 24 μM, where **5a** was the most potent compound with an IC_{50} value of 2.83 μM and **5g** the most potent thiohydantoin derivative with an IC_{50} value of 5.10 μM. The thiazolidinedione derivative **5J** was the least active among all tested compounds. In most of cases, the hydantoin derivatives were more active than their thiohydantoin and thiazolidinedione congeners *e.g.* **5a**, **d** and **e** *versus* **5f**, **h** and **i**. In addition the hydantoins were more active than their thiazolidinedione derivative **5c** *versus* **j**. This indicates the importance of the nature of the core ring on growth



i) $NaBH_4$, $CoCl_2$, CH_3COOH, s: iPrOH, s: DMF, overnight, rt. ii) 3,4-Difuorobenzyl chlorides or 3,4-difluorobenzoyl chloride, K_2CO_3, C: KI, s: DMF, 45 min, reflux.

Chart 2

Table 1. The Growth Inhibitory Activity of the Synthesized Compounds against Wild Type HCT116 Cell Line

Compound	IC_{50} HCT116 (μM)	Compound	IC_{50} HCT116 (μM)
5a	2.8	**5g**	5.1
5b	3.7	**5h**	9.3
5c	5.5	**5i**	13.2
5d	5.0	**5j**	19.8
5e	7.8	**7**	24.0
5f	7.4	**8**	12.2
5g	5.1	Nutlin-3	2.0

Table 2. The Activity of the Most Potent Compounds against Different Cell Lines

Compound	IC_{50} (μM)		
	HCT116	SW480	H1299
5a	2.8	6.7	9.2
5b	3.7	n.d	11.3
5d	5.0	13.0	n.d
5g	5.1	5.7	16.6
Nutlin-3	2.0	16.0	22.2

inhibition of cancerous cell line HCT116.

In our attempt to test the selectivity across various cancer cell lines with various expression levels of p53; the most potent compounds **5a**, **b**, **d**, **g** were further tested against p53-mutated cell lines; human adenocarcinoma strain (SW480) and p-53 null human non-small cell lung carcinoma cell line (H1299). The results in Table 2 showed moderate selectivity of the tested compounds towards the HCT116 cell line relative to SW480 and H1299 cell lines with selectivity index (SI) of 2.36 and 3.26 for compound **5a**. Nutlin-3 was the most selective compound with SI of 8.00 and 11.11, respectively.

In order to elucidate the p53-dependent mode of action, the most potent and selective compound (**5a**) was further tested against ($p53^{-/-}$) HCT116, ($p53^{-/-}$) mouse embryonic fibroblasts (MEF), wild type ($p53^{+/+}$) HCT116 and wild type ($p53^{+/+}$) MEF. These isogenic cell lines are commonly used to show the p53/MDM2-dependent mode of action.[20] Indeed, compound **5a** and Nutlin-3 potently inhibited the growth of HCT116 and MEF wild type p53 cell lines but showed greatly reduced activity in the same cell lines with p53 deletion as shown in Fig. 2, supporting the hypothesis that the cell growth inhibitory activity of both **5a** and Nutlin-3 is p53-dependent.

Additionally, a p53 translocation assay was performed using immunocytochemical staining and high content screening. Selection of the tested compounds **5a**, **b**, **g** were based on activity against wild type ($p53^{+/+}$) HCT116 cell line using Nutlin-3 as a positive control. From the results shown in Table 3, it's clear that compounds **5a** and **g** induced a dose-dependent increase in the nuclear p53 at lower concentrations which inhibit growth. For compound **5a**, this effect apparently reached a plateau between 5.56 and 16.67 μM, which might be attributed to the poor solubility and high lipophilicity of the compound; however, compound **5g** showed its strongest effect at 50 μM. The highest increase in nuclear p53 was observed after the treatment with **5a** at 16.67 μM, which is consistent with results obtained from cellular growth assays suggesting that the dominant mode of action for compound **5a** might be through inhibiting p53 stability. Western blotting data supported these findings and further showed that p53 accumulation induced by **5a** was accompanied by up-regulation of MDM2 and p21 proteins in wild type MEF and HCT116 cells (data represented in Fig. 3), all these data confirm that the mode of action of **5a** is p53-dependent through inhibition of the protein–protein interaction between p53 and its oncoproteins MDM2/*x*.

In an attempt to further explore the mode of action of **5a**; an assessment of the binding affinity characteristics of the compound towards MDM2 was examined *via* competitive fluorescence polarization assay described by Vogel *et al.*[21] K_d values were calculated from IC_{50} values using equations described by Nikolovska-Coleska *et al.*[22] Nutlin-3 was used as control, and as expected, Nutlin-3 disrupted these complexes with K_d values for MDM2 and MDM4 that were similar to growth inhibitory IC_{50} values for HCT116 and SW480 cells, respectively.

In contrast, **5a** displayed approximately 14-fold difference between K_d values and growth inhibitory IC_{50} for the same pairs (MDM2 *vs.* HCT116 and MDM4 *vs.* SW480). This is consistent with the expected mechanism for this and a similar

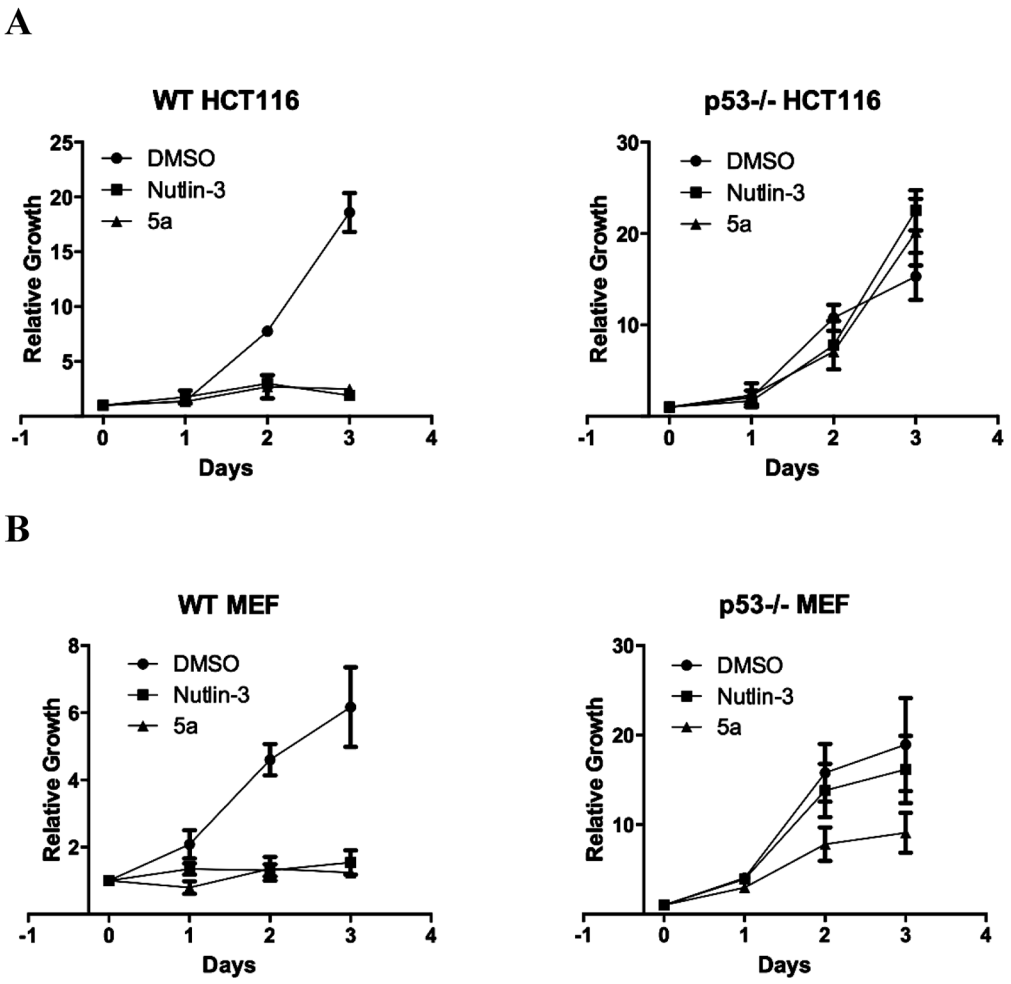


Fig. 2. Compound **5a** Exhibits p53 Dependent Inhibition of Cell Proliferation in Both HCT116 (A) and MEF Cells (B)

Cell proliferation in the presence of DMSO, 10 μM Nutlin-3, or 10 μM compound **5a** were completed using the BioRad TC10 Automated Cell Counter. Curves are the combination of multiple experiments MEF (n=4) and HCT116 (n=2). Relative growth was calculated as current cell number divided by Day 0 cell number. Error bars represent S.E.M.

Table 3. p53 Translocation Assay

Compound conc.	Fold change in nuclear p53 staining *vs.* DMSO control			
	50.00 μM	16.67 μM	5.56 μM	1.85 μM
5a	4.24±1.63	5.56±0.87	3.85±0.40	1.64±0.24
5b	1.10±0.25	1.48±0.28	1.17±0.23	0.92±0.15
5g	4.87±2.39	2.06±0.41	1.21±0.23	1.06±0.43
Nutlin-3	13.96±0.1 (30 μM)	8.77±0.98 (10 μM)	3.87±0.17 (3.3 μM)	1.93±0.24 (1.1 μM)

scaffold,[19] which was shown to affect p53 stability through MDM2 and MDM*x* homo- and heterodimerization rather than direct binding to MDM2 (results shown in Table 4). However, we also cannot completely rule out that affinity to other targets could contribute to the observed cellular profile *e.g.* while **5g** and **5a** have very similar IC_{50} in anti-proliferation assays, **5g** barely affects the expression of MDM2 and p21.

In summary, compound **5a** showed considerable growth inhibitory activity, while biochemical and biological assays showed that **5a** disrupts the MDM2/*x*–p53 interaction and activates the p53 pathway in cells with wild-type p53 leading to cell cycle arrest in both normal and tumor cells.

In conclusion, indolylhydantoin seems to be a promising scaffold for MDM2/MDM*x* inhibition.

Experimental

Chemistry General Solvents and reagents were obtained from commercial suppliers and used as received. A Bruker DRX 300 spectrometer was used to obtain ^{1}H- and ^{13}C-NMR spectra. The chemical shifts are referenced to the residual protonated solvent signals or tetramethylsilane (TMS) was used as a reference. Electrospray LC-MS analysis was performed using a TSQ quantum (Thermo Electron Corporation) instrument prepared with a triple quadrupole mass detector (ThermoFinnigan) and an electrospray ionization (ESI) source. All samples were inserted using an autosampler (Surveyor, ThermoFinnigan) by an injection volume of 10 μL. The MS detection was determined using a source collision induced dissociation (CID) of 10 V and carried out at a spray

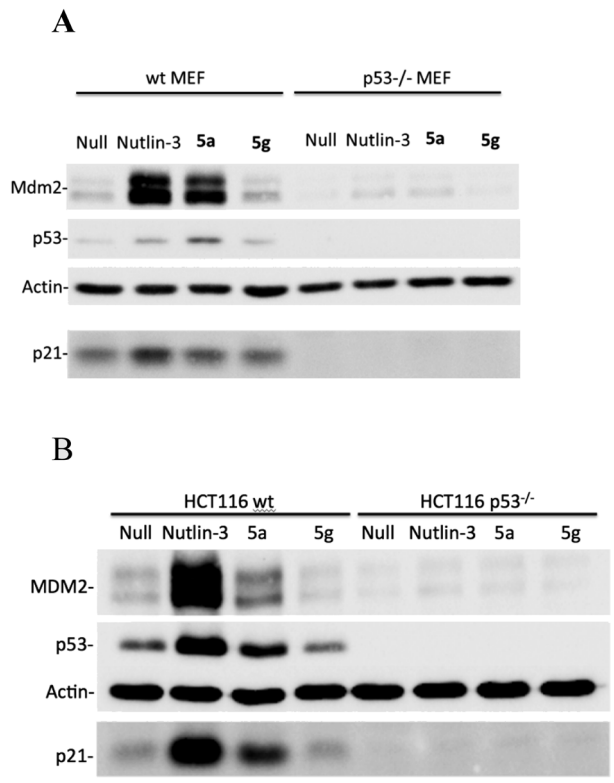


Fig. 3. Western Blotting Analysis for MDM2, p53 and p21 Proteins in Wild Type and ($p53^{-/-}$) MEF (A) and HCT116 Cells (B) When Treated with 10 μM Nutlin-3, 10 μM **5a** or 10 μM **5g** and Incubated for 24 h

Table 4. Fluorescence Polarization Assay

	MDM2			MDM*x*		
	IC_{50} (μM)	S.E. (μM)	K_d (μM)	IC_{50} (μM)	S.E. (μM)	K_d (μM)
5a	160	5.5	41.2	330	14.0	90.3
Nutlin-3	2.0	6.5	0.5	54.2	8.7	14.8

voltage of 4.2 kV, a nitrogen sheath gas pressure of 4.0×10^5 Pa, a capillary temperature of 400°C, a capillary voltage of 35 V and an auxiliary gas pressure of 1.0×10^5 Pa. The stationary phase used was an RP C18 NUCLEODUR 100-3 (125×3 mm) column (Macherey–Nagel). The solvent system consisted of water containing 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA in acetonitrile (B). HPLC method: flow rate 400 μL/min. The percentage of B started at an initial of 5%, was increased up to 100% during 16 min, kept at 100% for 2 min, and flushed back to 5% in 2 min.

General Procedure of Vilsmeier–Haack Reaction A solution of 6-chloroindole (30 mmol) in *N*,*N*-dimethylformamide (DMF) (5 mL) was added dropwise to a solution of phosphorus oxychloride (44 mmol) in DMF (32 mL) which had been stirred for 1.5 h at 0°C. The reaction mixture was warmed to room temperature and stirred for 2 h. The solution with the precipitate formed was poured on ice and basified with solid KOH and left overnight. The precipitate was filtered and washed with a mixture of 10% ethylacetate in petroleum benzene several times, then left to dry to give the aldehyde.

6-Chloro-1*H*-indole-3-carbaldehyde (**3**)

Synthesized according to the general procedure of Vilsmeier–Haack reaction; light brick red solid; yield 4.04 g (75%).

General Procedure of Knoevenagel Condensation Imidazolidine-2,4-dione or its appropriate sulfur-isostere (10.0 mmol) was dissolved in 10 mL of water or ethanol at 70°C with stirring. After complete dissolution, the pH of the mixture was adjusted to 7.0 with saturated $NaHCO_3$ solution. Ethanolamine (0.9 mL) was added to the reaction mixture, and the temperature was increased to 90°C by use of an oil bath. To this, an equimolar quantity of 6-chloro-1*H*-indole-3-carbaldehyde (**3**) (10.0 mmol) solution in 10 mL of alcohol was added dropwise with continuous stirring. The temperature was raised to 120°C and kept under reflux at that temperature overnight. The mixture was cooled, the precipitate was filtered and washed with water in order to remove the soluble impurities, then left to dry.

5-((6-Chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**)

Synthesized according to the general procedure of Knoevenagel condensation using imidazolidine-2,4-dione and 6-chloro-1*H*-indole-3-carbaldehyde (**3**); yellowish white solid; yield: 1.60 g (61%); ^{1}H-NMR (300 MHz, dimethyl sulfoxide (DMSO)) δ: 12.29 (s, 1H), 11.97 (s, 1H), 10.49 (s, 1H), 8.19 (s, 1H), 7.82 (d, J=8.6 Hz, 1H), 7.48 (d, J=1.9 Hz, 1H), 7.13 (dd, J=8.5, 1.9 Hz, 1H), 6.88 (s, 1H); ^{13}C-NMR (75 MHz, DMSO) δ: 164.49, 153.42, 136.18, 128.09, 127.06, 125.67, 122.56, 120.52, 119.73, 111.52, 108.46, 103.10; MS ESI: m/z=385.71 $(M)^+$; purity: 97%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-2-thioxoimidazolidin-4-one (**4b**)

Synthesized according to the general procedure of Knoevenagel condensation using 2-thioxoimidazolidin-4-oneand 6-chloro-1*H*-indole-3-carbaldehyde (**3**); redsolid; yield: 1.35 g (49%); ^{1}H-NMR (300 MHz, DMSO) δ: 12.79 (s, 1H), 11.94 (s, 1H), 11.59 (s, 1H), 8.40 (s, 1H), 8.20 (d, J=8.5 Hz, 1H), 7.52 (d, J=1.9 Hz, 1H), 7.13 (dd, J=8.5, 1.9 Hz, 1H), 7.10 (s, 1H); ^{13}C-NMR (75 MHz, DMSO) δ: 169.90, 158.62, 136.79, 135.19, 132.96, 127.05, 125.36, 121.19, 120.73, 115.72, 111.78, 111.24.

5-((6-Chloro-1*H*-indol-3-yl)methylene)thiazolidine-2,4-dione (**4c**)

Synthesized according to the general procedure of Knoevenagel condensation using thiazolidine-2,4-dione and 6-chloro-1*H*-indole-3-carbaldehyde (**3**); yellow solid; yield: 1.25 g (45%); ^{1}H-NMR (300 MHz, DMSO) δ: 12.44 (s, 1H), 12.23 (s, 1H), 8.18 (s, 1H), 7.93 (d, J=8.6 Hz, 1H), 7.83 (s, 1H), 7.28 (dd, J=8.0, 1.1 Hz, 1H), 7.19 (dd, J=8.5, 1.4 Hz, 1H), ^{13}C-NMR (75 MHz, DMSO) δ: 167.56, 165.77, 137.28, 131.63, 128.26, 126.35, 126.02, 121.91, 120.60, 114.97, 112.56, 111.01.

General Procedure of Benzylation To a suspension of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione or its appropriate sulfur-isostere (**4a**–**c**) (10 mmol), K_2CO_3 (40 mmol) and KI (0.6 mmol) in dry DMF (20 mL), the appropriate benzyl chloride was added (12 mmol). The resulting mixture was refluxed for 45 min. The reaction was stopped by adding water, and then the mixture was extracted with EtOAc (3×20 mL). The combined organic phases were washed with brine, dried over anhydrous Mg_2SO_4, filtered and concentrated. The crude residue was then purified by column chromatography on silica gel.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(4-chlorobenzyl)-imidazolidine-2,4-dione (**5a**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione

(**4a**) and 1-chloro-4-(chloromethyl)benzene according to the general procedure for benzylation; yellowish white solid; yield: 1.55 g (40%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.97 (s, 1H), 10.49 (s, 1H), 8.19 (s, 1H), 7.82 (d, J=8.6 Hz, 1H), 7.48 (d, J=1.9 Hz, 1H), 7.44–7.38 (m, 2H), 7.37–7.29 (m, 2H), 7.13 (dd, J=8.5, 1.9 Hz, 1H), 6.88 (s, 1H), 4.68 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 163.59, 154.42, 136.18, 135.75, 132.08, 129.33, 128.54, 128.07, 127.06, 125.67, 122.56, 120.52, 119.73, 111.52, 108.46, 103.00, 40.58; MS (ESI): m/z=387.39 $(M+H)^+$; purity: 98.2%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3-chlorobenzyl)-imidazolidine-2,4-dione (**5b**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) and 1-chloro-3-(chloromethyl)benzene according to the general procedure for benzylation; yellowish white solid; yield: 1.69 g (44%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.97 (s, 1H), 10.50 (s, 1H), 8.19 (s, 1H), 7.83 (d, J=8.5 Hz, 1H), 7.48 (d, J=1.8 Hz, 1H), 7.43–7.32 (m, 3H), 7.31–7.23 (m, 1H), 7.13 (dd, J=8.5, 1.8 Hz, 1H), 6.89 (s, 1H), 4.69 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 163.62, 154.40, 139.21, 136.18, 133.13, 130.53, 128.10, 127.47, 127.28, 127.07, 126.06, 125.68, 122.53, 120.53, 119.74, 111.53, 108.47, 103.11, 40.67; MS (ESI): m/z=387.68 $(M+H)^+$; purity: 96%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-dichlorobenzyl)-imidazolidine-2,4-dione (**5c**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) and 1,2-dichloro-4-(chloromethyl)benzene according to the general procedure for benzylation; yellowish white solid; yield: 2.00 g (49%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.97 (s, 1H), 10.50 (s, 1H), 8.19 (s, 1H), 7.82 (d, J=8.6 Hz, 1H), 7.61 (d, J=8.3 Hz, 1H), 7.58 (d, J=2.0 Hz, 1H), 7.48 (d, J=1.9 Hz, 1H), 7.29 (dd, J=8.3, 2.0 Hz, 1H), 7.13 (dd, J=8.5, 1.9 Hz, 1H), 6.88 (s, 1H), 4.69 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 163.60, 154.34, 137.85, 136.18, 131.09, 130.80, 130.13, 129.54, 128.11, 127.76, 127.07, 125.67, 122.53, 120.53, 119.73, 111.53, 108.46, 103.15, 40.69; MS (ESI): m/z=419.50 $(M-H)^+$; purity: 96.9%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-difluorobenzyl)-imidazolidine-2,4-dione (**5d**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) and 4-(chloromethyl)-1,2-difluorobenzene according to the general procedure for benzylation; faint yellow solid; yield: 1.74 g (45%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.97 (s, 1H), 10.49 (s, 1H), 8.19 (s, 1H), 7.82 (d, J=8.5 Hz, 1H), 7.48 (d, J=1.8 Hz, 1H), 7.46–7.33 (m, 2H), 7.13 (dd, J=8.5, 1.9 Hz, 2H), 6.88 (s, 1H), 4.68 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 163.60, 162.32, 154.38, 150.87, 136.18, 134.45, 128.08, 127.07, 125.67, 124.25, 122.58, 120.52, 119.73, 117.64, 116.67, 111.53, 108.47, 103.05, 40.58; MS (ESI): m/z=387.77 $(M)^+$; purity: 98.5%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(4-(trifluoromethyl)-benzyl)imidazolidine-2,4-dione (**5e**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) and 1-(chloromethyl)-4-(2,2,2-trifluoroethyl)-benzeneaccording to the generalprocedure for benzylation; yellow solid; yield: 2.31 g (55%); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 11.98 (s, 1H), 10.53 (s, 1H), 8.20 (s, 1H), 7.83 (d, J=8.5 Hz, 1H), 7.72 (d, J=8.2 Hz, 2H), 7.52 (d, J=8.1 Hz, 2H), 7.48 (d, J=1.7 Hz, 1H), 7.13 (dd, J=8.5, 1.8 Hz, 1H), 6.89 (s, 1H), 4.78 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 163.63, 154.40, 141.44, 136.19, 128.08, 128.07, 127.08, 125.99, 125.67, 125.50, 122.39, 122.53, 120.53, 119.73, 111.53, 108.46, 103.14, 40.83; MS (ESI): m/z=419.68 $(M)^+$; purity: 96%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(4-chlorobenzyl)-2-thioxoimidazolidin-4-one (**5f**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)-2-thioxoimidazolidin-4-one (**4b**) and 1-chloro-4-(chloromethyl)benzene according to the general procedure for benzylation; orange solid; yield: 1.52 g (38%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.94 (s, 1H), 11.59 (s, 1H), 8.40 (s, 1H), 8.20 (d, J=8.5 Hz, 1H), 7.59–7.53 (m, 2H), 7.52 (d, J=1.9 Hz, 1H), 7.46–7.36 (m, 2H), 7.13 (dd, J=8.5,1.9 Hz, 1H), 7.10 (s, 1H), 4.59 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 169.91, 158.72, 136.77, 136.62, 135.19, 132.96, 132.00, 130.73, 128.48, 127.05, 125.36, 121.19, 120.73, 115.72, 111.78, 111.24, 32.47; MS (ESI): m/z=403.68 $(M+H)^+$; purity: 95%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-dichlorobenzyl)-2-thioxoimidazolidin-4-one (**5g**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)-2-thioxoimidazolidin-4-one (**4b**) and 1,2-dichloro-4-(chloromethyl)benzene according to the general procedure for benzylation; dark yellow solid; yield: 1.70 g (39%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.95 (s, 1H), 11.62 (s, 1H), 8.41 (s, 1H), 8.19 (d, J=8.5 Hz, 1H), 7.84 (d, J=1.9 Hz, 1H), 7.63–7.53 (m, 2H), 7.52 (d, J=1.9 Hz, 1H), 7.15 (dd, J=8.5, 1.9 Hz, 1H), 7.08 (s, 1H), 4.59 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 169.88, 158.50, 139.01, 136.83, 135.10, 132.92, 130.94, 130.88, 130.64, 129.95, 129.20, 127.08, 125.34, 121.20, 120.77, 115.90, 111.79, 111.21, 31.91; MS (ESI): m/z=437.90 $(M+H)^+$; purity: 98%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-difluorobenzyl)-2-thioxoimidazolidin-4-one (**5h**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)-2-thioxoimidazolidin-4-one (**4b**) and 4-(chloromethyl)-1,2-difluorobenzene according to the general procedure for benzylation; orange solid; yield: 1.60g (40%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.95 (s, 1H), 11.61 (s, 1H), 8.41 (s, 1H), 8.21 (d, J=8.6 Hz, 1H), 7.67–7.57 (m, 1H), 7.52 (d, J=1.9 Hz, 1H), 7.44–7.33 (m, 2H), 7.16 (dd, J=8.5, 1.9 Hz, 1H), 7.09 (s, 1H), 4.59 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 169.91, 158.60, 151.40, 147.38, 136.82, 135.42, 135.13, 132.94, 127.08, 125.80, 125.35, 121.22, 120.75, 117.73, 117.73, 115.82, 111.79, 111.24, 32.11; MS (ESI): m/z=403.60 $(M)^+$; purity: 96%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-2-thioxo-3-(4-(trifluoromethyl)benzyl)imidazolidin-4-one (**5i**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)-2-thioxoimidazolidin-4-one (**4b**) and 1-(chloromethyl)-4-(2,2,2-trifluoroethyl)benzene according to the general procedure for benzylation; brick red solid; yield: 1.74 g (40%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.95 (s, 1H), 11.63 (s, 1H), 8.39 (d, J=2.7 Hz, 1H), 8.19 (d, J=8.6 Hz, 1H), 7.80–7.68 (m, 4H), 7.52 (d, J=1.8 Hz, 1H), 7.14 (dd, J=8.5, 1.9 Hz, 1H), 7.06 (s, 1H), 4.69 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 169.90, 158.56, 142.61, 136.78, 135.13, 133.01, 129.63, 127.92, 127.06, 125.99, 125.34, 122.39, 121.20, 120.73, 115.86, 111.78, 111.22, 32.58; MS (ESI): m/z=435.67 $(M)^+$; purity: 96%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-dichlorobenzyl)-thiazolidine-2,4-dione (**5j**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)thiazolidine-2,4-dione (**4c**) and 1,2-dichloro-4-(chloromethyl)benzene according to the general procedure for benzylation; yellow solid; yield: 1.22 g (28%); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 12.23 (s, 1H), 8.18 (s, 1H), 7.93 (d, J=8.6 Hz, 1H), 7.83 (s, 1H), 7.60 (d, J=8.3 Hz, 2H), 7.53 (d, J=8.0, 1H), 7.28 (dd, J=8.0, 1.1 Hz, 1H), 7.19 (dd, J=8.5, 1.4 Hz, 1H), 4.82 (s, 2H); ^{13}C-NMR (75 MHz, DMSO) δ: 167.56, 165.77, 137.28, 137.12, 131.63, 131.34, 130.93, 130.52, 130.32, 128.45, 128.26, 126.35, 126.02, 121.91, 120.60, 114.97, 112.56, 111.01, 43.86; MS (ESI): m/z=437.62 $(M)^+$; purity: 97%.

5-((6-Chloro-1*H*-indol-3-yl)methyl)imidazolidine-2,4-dione (**6**)

Synthesized by a reaction between a solution of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) (2 mmol) in a mixed solvent of DMF–isopropanol (1 : 2, 60 mL), $CoCl_2$ (12 mmol), acetic acid (90 mmol) and $NaBH_4$ (120 mmol) portion wise. The mixture was stirred at room temperature overnight and then diluted with ethyl acetate (100 mL). The mixture was washed sequentially with saturated $NaHCO_3$ (50 mL), 1 N HCl (50 mL), saturated NaCl (50 mL) and then dried over anhydrous $MgSO_4$, filtered and concentrated. The crude residue was then purified by column chromatography on silica gel; white powder; yield: 0.13 g (25%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.11 (s, 1H), 11.01 (s, 1H), 8.37 (s, 1H), 7.51 (d, J=8.5 Hz, 1H), 7.36 (d, J=1.8 Hz, 1H), 6.92 (dd, J=8.5, 1.9 Hz, 1H), 6.35–6.27 (m, 1H), 4.46 (t, J=4.1 Hz, 1H), 3.18 (dd, J=14.9, 4.3 Hz, 1H), 3.06 (dd, J=14.9, 4.3 Hz, 1H); ^{13}C-NMR (75 MHz, DMSO) δ: 174.02, 156.51, 136.16, 127.27, 125.59, 122.93, 120.09, 118.68, 110.85, 107.58, 57.18, 25.96.

5-((6-Chloro-1*H*-indol-3-yl)methyl)-3-(3,4-difluorobenzyl)-imidazolidine-2,4-dione (**7**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methyl)-2-thioxoimidazolidin-4-one (**6**) and 4-(chloromethyl)-1,2-difluorobenzene according to the general procedure for benzylation; white solid; yield: 1.55 g (40%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.01 (s, 1H), 8.37 (s, 1H), 7.51 (d, J=8.5 Hz, 1H), 7.36 (d, J=1.8 Hz, 1H), 7.11 (d, J=2.3 Hz, 1H), 7.04 (dt, J=10.8, 8.5 Hz, 1H), 6.92 (dd, J=8.5, 1.9 Hz, 1H), 6.76 (ddd, J=11.3, 7.8, 2.0 Hz, 1H), 6.35–6.27 (m, 1H), 4.46 (t, J=4.1 Hz, 1H), 4.34 (d, J=15.7 Hz, 1H), 4.22 (d, J=15.7 Hz, 1H), 3.18 (dd, J=14.9, 4.3 Hz, 1H), 3.06 (dd, J=14.9, 4.3 Hz, 1H); ^{13}C-NMR (75 MHz, DMSO) δ: 173.62, 156.27, 151.07, 147.10, 136.16, 133.95, 127.27, 125.90, 125.59, 122.93, 120.09, 118.68, 116.90, 115.68, 110.85, 107.58, 57.18, 41.12, 25.96; MS (ESI): m/z=389.71 $(M)^+$; purity: 97.6%.

5-((6-Chloro-1*H*-indol-3-yl)methylene)-3-(3,4-difluorobenzoyl)-imidazolidine-2,4-dione (**8**)

The title compound was prepared by reaction of 5-((6-chloro-1*H*-indol-3-yl)methylene)imidazolidine-2,4-dione (**4a**) and 3,4-difluorobenzoyl chloride according to the general procedure for benzylation; dark yellow solid; yield: 3.00 g (75%); ^{1}H-NMR (300 MHz, DMSO) δ: 11.33 (s, 1H), 10.58 (s, 1H), 8.36 (d, J=1.8 Hz, 1H), 8.14 (s, 1H), 8.10–7.99 (m, 2H), 7.78 (dd, J=8.8, 4.5 Hz, 2H), 7.52 (dd, J=8.5, 1.9 Hz, 1H), 6.64 (s, 1H); ^{13}C-NMR (75 MHz, DMSO) δ: 166.70, 165.43, 155.96, 152.54, 149.74, 142.56, 136.04, 131.14, 130.69, 129.01, 128.83, 127.90, 125.15, 121.06, 119.78, 118.65, 116.11, 114.52, 97.39; MS (ESI): m/z=401.71 $(M)^+$; purity: 98%.

Cell Culture Cancer cell lines cultured included wild-type p53 cell line (HCT-116), and p53 null cell line (H1299) which were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in a 37°C humidified incubator with 5% CO_2 with the same medium (RPMI-1640 supplemented with 5% fetal bovine serum), and passaged twice weekly. Only cultures exhibiting greater than 95% viability were used in growth inhibition experiments (determined by trypan blue exclusion).

Growth Inhibition Assay Cells were seeded in 96-well tissue culture-treated assay plates at a density of 1.5×10^4 cells/cm^2, then allowed to attach overnight before addition of experimental compounds. Compounds were dissolved in DMSO, then diluted to the final concentration indicated so as to ensure DMSO concentration less than 0.2%. After treatment with either a single screening concentration or a titration series of concentrations of compounds, cells were incubated for an additional 72 h. Relative cell growth was determined by addition of PromegaCellTiterGlo luciferase-based assay of ATP content. The resultant luminescence was measured, and each data set was analyzed using DMSO (vehicle control) as a baseline value for growth inhibition. GraphPad Prism software was used to develop dose–response curves and IC_{50} values for active compounds.

Immunofluorescent Labeling p53 Cells were seeded in 96-well tissue culture-treated assay plates at a density of 1.5×10^4 cells/cm^2, then allowed to attach overnight before addition of experimental compounds.

Compound Treatment: Compounds were dissolved in DMSO, then diluted to the final concentration indicated so as to ensure DMSO concentration less than 0.2%. Experimental compounds were added to cells in equal volume, maintaining equal concentration of DMSO vehicle among all tested compound concentrations. After 20 h treatment period, cells were fixed with 4% formaldehyde (Sigma-Aldrich) for 20 min at room temperature.

Fixative was removed, and samples washed thrice in phosphate buffered saline (PBS). Nonspecific binding sites were blocked and cells permeabilized by 60 min incubation with 5% fetal bovine serum and 0.3% Triton X-100 in 0.9% PBS. Samples were incubated overnight at 4°C with anti-p53 rabbit monoclonal antibody (Cell Signaling Technology) diluted 1 : 1600 in antibody dilution buffer (1% bovine serum albumin and 0.3% Triton X-100 in PBS). Samples were washed thrice in PBS. Samples were then incubated 2 h at room temperature with Alexa Fluor 488 anti rabbit polyclonal antibody conjugate (Life Technology) diluted 1 : 500 in antibody dilution buffer, then washed thrice in PBS. Nuclei were stained with DAPI (Sigma-Aldrich; 1 μg/mL) for 30 min at room temperature.

Imaging Assay: Samples were imaged using the Molecular Devices ImageXpressMicroXL high content screening instrument fitted with a 20× ELWD Nikon objective lens. DAPI and AlexaFluor 488 fluorophores were visualized using 405ex/450em and 488ex/525em filter sets, respectively. Four identically located fields from within each well were captured in each color channel. MetaXpress image analysis software was used to segment images and measure p53-associated fluorescent intensity within the nuclear and cytoplasmic region of each cell. Average intensity values, cell number, and nuclear to cytoplasmic intensity ratio for each well are reported.

Western Blotting After lysing cells in 0.5% NP-40 buffer, the lysates were resolved on a 12.5% polyacrylamide gel and then transferred to a 0.2-μm nitrocellulose membrane. Membranes were blocked for at least 30 min in PBS blocking buffer with 0.1% Tween-20 (PBST) and 5% non-fat dried milk. Membranes were incubated for 2 h to overnight with the appropriate primary antibody, incubated for 1–2 h in secondary horseradish peroxidase (HRP)-conjugated antibody, and exposed with Supersignal West Pico or Dura reagent (Pierce). Mouse monoclonal MDM2 (2A10, Calbiochem), p53 (NCL-505, Leica), actin (MAB1501, Chemicon International), and goat polyclonal p21 (C-19; Santa Cruz) antibodies were purchased commercially.

Fluorescence Polarization Assay The assay was performed as previously described by Vogel *et al.*[21] Briefly, the association of a FAM labeled peptide (derived from the amino terminus of p53 from recombinant MDM2 (AA16–116) or MDM4 (AA2–125) was measured by anisotropy. Dissociation constants were determined by direct competition of the tested compounds.

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Conflict of Interest The authors declare no conflict of interest.

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