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Synthesis and evaluation of spiroisoxazoline oxindoles as anticancer agents

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ABSTRACT

Restoring p53 levels through disruption of p53–MDM2 interaction has been proved to be a valuable approach in fighting cancer. We herein report the synthesis and evaluation of eighteen spiroisoxazoline oxindoles derivatives as p53–MDM2 interaction inhibitors. Seven compounds showed an antiproliferative profile superior to the p53–MDM2 interaction inhibitor nutlin-3, and induced cell death by apoptosis. Moreover, proof-of-concept was demonstrated by inhibition of the interaction between p53 and MDM2 in a live-cell bimolecular fluorescence complementation assay.

1. Introduction

Tumor suppressor p53 was discovered over 30 years ago and since then it has emerged as a key protein in many biological processes. p53 plays a pivotal role in the regulation of cell cycle, apoptosis, DNA repair, senescence and angiogenesis, and consequently carcinogenesis.[1,2] It is now believed that loss of p53 function occurs in virtually all cancers. Indeed, in approximately 50% of human cancers, the *TP53* gene is mutated or deleted. In tumors with wild type p53, protein function is usually inactivated by overexpression of negative regulators, primarily the murine double minute-2 (MDM2), which form an autoregulatory feedback loop.[3]

Since increased levels of p53 inactivator MDM2 are present in 7% of all cancers, targeting the p53–MDM2 interaction to reactivate p53 has emerged as a promising new cancer therapeutic strategy.[4] Furthermore, studies using p53–MDM2 interaction inhibitors showed that in normal cells the activation of p53 induces cell cycle arrest but not cell death, revealing a selective apoptotic effect on tumor cells.[5]

In the bound conformation, the α-helix of the transactivation domain of p53 projects residues Phe19, Trp23 and Leu26 into a deep hydrophobic cleft in the MDM2 protein, representing the critical residues for binding between these two proteins to occur.[6]

In recent few years, several compounds families were designed and developed as modulators of p53.[7–29] Three classes of p53–MDM2 interaction inhibitors are of particular importance: *cis*-imidazolines (e.g., nutlin-3a, Fig. 1),[9] benzodiazepinediones (e.g., TDP665759, Fig. 1),[30] and spiro-oxindoles (e.g., MI-219, Fig. 1).[31] Recently a new family containing a piperidone ring as emerged as a promising new scaffold (e.g., AM-8553, Fig. 1).[17] Each

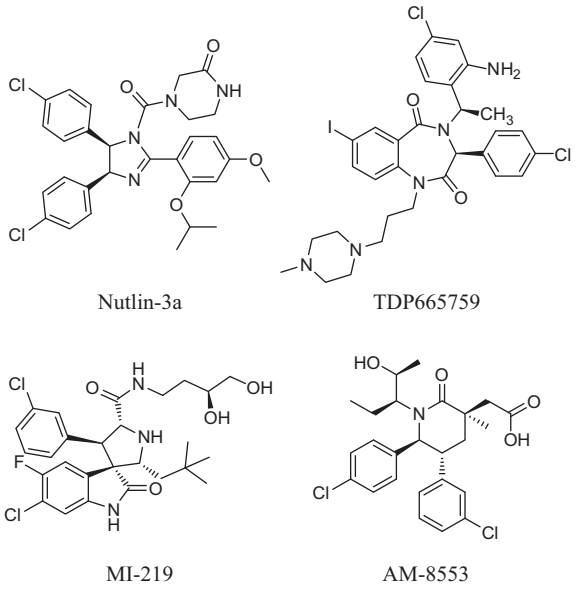

Figure 1. Representative p53–MDM2 interaction inhibitors.

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of them detains a unique, rigid heterocyclic scaffold, from which three lipophilic groups are projected into p53 pocket in MDM2 mimicking the three pivotal Phe19, Trp23 and Leu26. All the interactions are mostly hydrophobic, with potency increasing by introduction of halide-substituted aromatic groups.[32]

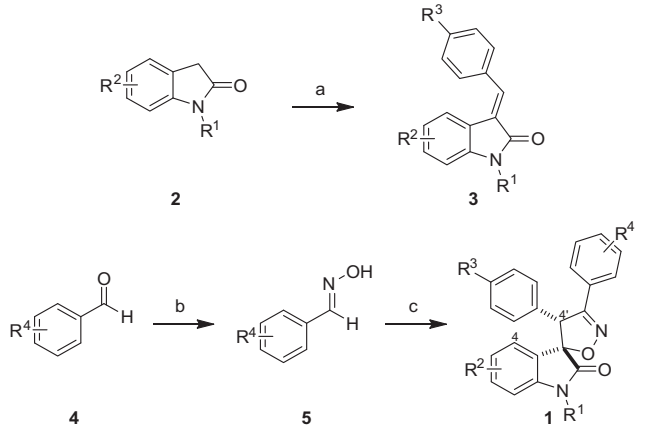
However, the goal of developing a p53 reactivating cancer therapy through inhibition of p53–MDM2 interaction is still in its infancy, with only a few candidates entering clinical trials.[33] In spite of this, more potent and selective p53–MDM2 interaction inhibitors are still largely required.

Therefore, we synthesized a library of novel spiroisoxazoline oxindoles, to be evaluated as p53–MDM2 interaction inhibitors in cell-based assays. The oxindole moiety would mimic the indole side chain of Trp23 of p53 and the isoxazoline ring, would act as the rigid heterocyclic scaffold. In addition, due to the fact that mimicking p53 entails three hydrophobic moieties, we synthesized 18 derivatives with different aromatic side chains attached to the isoxazoline moiety.

2. Results and discussion

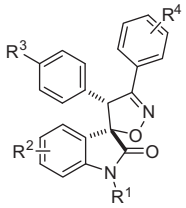
Spiroisoxazoline oxindoles **1** (Scheme 1) were synthesized by 1,3-dipolar cycloaddition between 3-methylene indolin-2-ones 3 and nitrile oxides. Aldoximes **5** were synthesized from the corresponding aldehyde **4** by reaction with hydroxylamine.[34] Then, aldoximes were reacted with NCS to form hydroximoyl chlorides. With exception of compound **1e**, the next step proceeded without isolation of chlorooxime by addition of compound **3** to form the final spirooxindole compound. In these cases, the 1,3-dipoles were generated in situ through dehydrohalogenation of the chlorooxime in the presence of triethylamine. Intermediates **3** were synthesized by aldolic condensation of substituted indolin-2-ones with different aromatic aldehydes in the presence of piperidine.[35] Compound **1e** was synthesized by reaction of the corresponding intermediate **3** with *N*-hydroxybenzimidoyl chloride in the presence of metallic Zinc.[36] The relative configuration was established by NMR comparisons with other spiroisoxazoline oxindoles, with published X-ray crystallography structure.[36,37] In this configuration the isoxazoline proton (H-4′) and the H-4 are more shielded in comparison to the alternative configuration, as observed by Risitano.[37] Furthermore, we observed NOE effect between H-4 and the *ortho*-protons of the aryl at C-4′, revealing spatially proximity and corroborating the established configuration.

We started by evaluating the antiproliferative activity in a human hepatocellular carcinoma cell line (HepG2) with wild type



Scheme 1. Synthesis of spiroisoxazoline oxindoles **1**. Reagents and conditions: (a) aromatic aldehydes, piperidine, EtOH, reflux, 76–99%; (b) $NH_2OH.HCl$, Na_2CO_3, H_2O, reflux, 87–98%; (c) 1. NCS, piridine, $CHCl_3$; 2. Compound **3**, Et_3N, $CHCl_3$, reflux, 43–76%.

Table 1
In vitro antiproliferative activities in HepG2 cell line



Compds	R^1	R^2	R^3	R^4	HepG2 GI_{50}[a] (μM)
1a	H	H	H	H	>100
1b	H	H	H	*p*-NO_2	52.14 ± 1.09
1c	H	H	H	*p*-OCH_3	77.77 ± 1.05
1d	H	H	OCH_3	H	>100
1e	CH_3	H	OCH_3	H	>100
1f	H	H	OCH_3	*p*-OCH_3	>100
1g	H	5-NO_2	H	H	83.59 ± 1.04
1h	H	5-NO_2	H	*p*-OCH_3	53.10 ± 1.14
1i	H	7-Cl	H	*p*-NO_2	55.48 ± 1.11
1j	H	7-Cl	H	*p*-OCH_3	55.79 ± 1.11
1k	H	6-Cl	H	H	37.07 ± 1.08
1l	H	6-Cl	H	*p*-OCH_3	38.14 ± 1.08
1m	H	6-Cl	H	*p*-NO_2	33.84 ± 1.11
1n	H	6-Cl	H	*p*-CH_3	29.11 ± 1.09
1o	H	6-Cl	H	*o*-OCH_3	35.70 ± 1.07
1p	H	6-Cl	OCH_3	*p*-OCH_3	>100
1q	H	6-Br	H	H	32.84 ± 1.07
1r	H	6-Br	H	*p*-OCH_3	31.69 ± 1.10
Nutlin-3					51.31 ± 1.04

[a] GI_{50} determined by the MTS method. Each value is the mean (GI_{50} ± SEM) of three independent experiments.

p53 (Table 1). Compound **1a** with no substituents in the three phenyl rings was not active. However, any substituent introduced at position R^2 and R^4 gave rise to active compounds (**1b–c**, **1g–o**, and **1q–r**). Introducing a methoxy group at position R^3 abrogates cytotoxicity, despite the pattern of substituents in other positions (**1d–f**, and **1p**).

The best compounds were obtained when a halogen was introduced at position 6 (Cl or Br) of the oxindole moiety (**1k–o**, and **1q–r**). In fact, a spiropyrrolidine oxindole derivative already published with co-crystal structure bound to MDM2[38] revealed that the oxindole moiety can perfectly mimic the indole side chain of Trp23 of p53. Furthermore, the strength of this interaction is increased by introducing a halogen at position 6 that can fill a small pocket not occupied by indole, corroborating our findings. Different substituents at R^4 in *para* position (methoxy, nitro and methyl) and *ortho*-methoxy are well tolerated, not affecting substantially potency. The best activity was found for compound **1n** (GI_{50} = 29.1 μM).

To investigate if cytotoxicity is mediated by p53, we tested all compounds more active than nutlin-3 (GI_{50} <50 μM) in three other cell lines with different p53 status: an isogenic pair of wild type p53 and null human colorectal cancer cell lines [HCT116 $p53^{(+/+)}$ and $p53^{(-/-)}$]; and a p53 mutant human colorectal adenocarcinoma cell line (SW620) (Table 2). All compounds showed only a marginal increase in potency in cell lines harboring wild type p53, revealing that p53 non-related effects are also contributing to cytotoxicity. However, due to similar response profile obtained for nutlin-3 in results assessed after 24 h, we further investigated if the compounds can in fact inhibit p53–MDM2 interaction. By applying a Venus-based bimolecular fluorescence complementation system methodology (BiFC) developed by our group,[39] we demonstrated that compound **1k** can inhibit p53–MDM2 interaction in the same extent as nutlin-3 (Fig. 2). Similar results were also obtained for compounds **1l** and **1n** (Supplementary data).

Table 2
In vitro antiproliferative activities

Compds	HepG2 GI_{50}[a] (μM)	HCT$p53^{(+/+)}$ GI_{50}[a] (μM)	HCT$p53^{(-/-)}$ GI_{50}[a] (μM)	SW620 GI_{50}[a] (μM)
1k	37.07 ± 1.08	39.80 ± 1.10	48.22 ± 1.13	48.86 ± 1.08
1l	38.14 ± 1.08	34.72 ± 1.14	40.14 ± 1.17	53.20 ± 1.10
1m	33.84 ± 1.11	32.37 ± 1.12	38.97 ± 1.14	33.52 ± 1.07
1n	29.11 ± 1.09	26.56 ± 1.07	30.64 ± 1.12	31.56 ± 1.05
1o	35.70 ± 1.07	30.51 ± 1.08	35.56 ± 1.10	36.74 ± 1.04
1q	32.84 ± 1.07	35.01 ± 1.07	40.55 ± 1.11	39.65 ± 1.07
1r	31.69 ± 1.10	33.38 ± 1.13	39.03 ± 1.18	40.36 ± 1.09
Nutlin-3	51.31 ± 1.04	39.65 ± 1.12	52.34 ± 1.15	57.04 ± 1.04

[a] GI_{50} determined by MTS method. Each value is the mean (GI_{50} ± SEM) of three independent experiments.

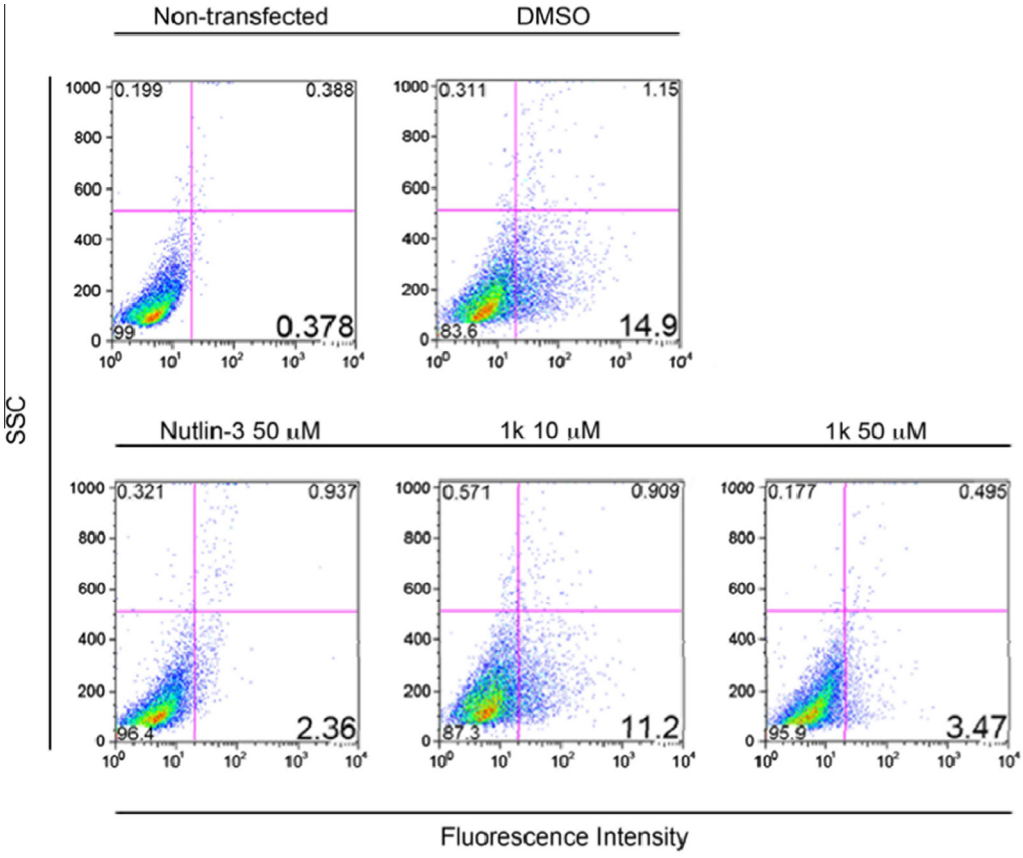


Figure 2. Compound **1k** decreases p53–MDM2 interaction by BiFC. HCT116 $p53^{(-/-)}$ cells were co-transfected with V1-p53/MDM2-V2 BiFC combination plasmids for 24 h. Vehicle, nutlin-3 (50 μM) and compound **1k** (10 and 50 μM) were included in the culture medium 4 h after transfection. Representative flow cytometry profiles of the disruption of V1-p53/MDM2-V2 complementation ($n = 3$).

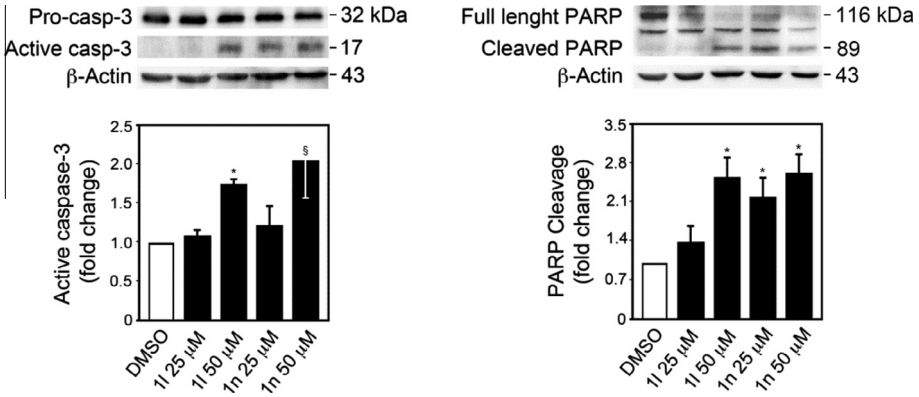


Figure 3. Compounds **1l** and **1n** induces caspase-3 activation and PARP cleavage. HCT116 $p53^{(+/+)}$ cells were incubated with vehicle or 25 and 50 μM of compounds **1l** and **1n**, for 24 h. Representative immunoblots of caspase-3 processing (*left*) and PARP cleavage (*right*) analyzed in whole cell extracts. Blots were normalized to endogenous β-actin. Data represent mean ± SEM of three independent experiments. $^{*}p$ <0.01 and $^{§}p$ <0.05 from control.

Inhibition of p53–MDM2 interaction will restore p53 in cancer cell lines leading to its activation, and consequently induce a p53-mediated signaling pathway that will culminate in cell death by apoptosis. The activation of caspase-3 upon cleavage by upstream proteases and subsequent cleavage of caspase-3 substrate PARP are considered reliable markers of the apoptotic process. In accordance, compounds **1l** and 1n induced a significant dose-dependent increase of cleaved PARP (p <0.01) and active caspase-3 (p <0.01 and p <0.05 for compound **1l** and **1n**, respectively) as detected by Western blotting (Fig. 3).

Chemical stability in pH 7.4 phosphate buffer and metabolic stability in human plasma and rat liver microsomes at 37 °C were evaluated for compounds **1l** and **1r**. Both compounds were stable in phosphate buffer and plasma for the duration of the assays (3 days). Compounds **1l** and **1r** exhibited degradation when incubated in rat microsomes with NADPH regenerating system, with half-lives of 3.15 ± 0.07 h and 3.62 ± 0.06 h, respectively, indicating moderate susceptibility towards co-factor dependent microsomal enzymes.

3. Conclusion

Eighteen spiroisoxazoline oxindole derivatives were synthesized with different substituents attached to the three phenyl rings to probe their capacity of inhibiting the p53–MDM2 interaction. Screening the compounds in a HepG2 cell line revealed that compounds with chloro or bromo at position 6 of the oxindole aromatic ring were more active than nutlin-3. The profile of inhibitory activity in cell lines with different p53 status was comparable to that of nutlin-3, revealing also p53-independent effects. In addition, compound **1k** showed inhibition of p53–MDM2 interaction in a cell-based bimolecular fluorescence complementation assay. Moreover, compounds **1l** and **1r** showed good stability in 7.4 phosphate buffer and plasma, and moderate susceptibility towards NADPH-dependent rat microsomal enzymes.

In conclusion, besides the potential use of the active compounds as molecular probes and possible anticancer agents, compound **1n** represent a useful lead compound for the development of more potent and selective p53–MDM2 interaction inhibitors.

4. Experimental

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Shimadzu FTIR Affinity-1 spectrophotometer and the UV spectra on a Shimadzu UV–Vis Recording Spectrophotometer UV-160. Elemental analysis (C, H, and N) were performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK) at Liquid Chromatography and Mass Spectrometry Laboratory, Faculty of Pharmacy of Lisbon University and also in a LECO model CHNS-932 elemental analyzer at the Unit Elemental Analysis, University of Santiago de Compostela, Spain. The 7 most active compounds also showed purity ⩾95% by analytical HPLC with absorbance at 260 nm. Merck Silica Gel 60 F254 plates were used for analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh) and Combi-Flash Rf from Teledyne ISCO (columns RediSep Rf, silica). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR). Data are reported as follows: chemical shift (δ), multiplicity (s: singlet, d: doublet, dd: doublet of doublet; ddd: doublet doublet of doublets, t: triplet, td: triplet of doublets, m: multiplet, br: broad), coupling constants (J) in Hertz and integration. ^{1}H and ^{13}C chemical shifts are expressed in ppm using the solvent as internal reference.

4.1.1. General preparation of derivatives 1a-d and 1f–r

To a stirred solution of *N*-chlorosuccinimide (3 equiv) and pyridine (0.3 equiv) in chloroform (2.5 mL/0.1 mmol of 3-methylene indolin-2-one) was added the appropriate aldoxime (3 equiv). The reaction mixture was stirred for 24 h at room temperature before addition of the appropriate 3-methylene indolin-2-one. The mixture was then heated to 50 °C and triethylamine (3.5 equiv) was added in a dropwise manner. After heating at refluxed for 26 h, the mixture was washed with brine (2×) and the aqueous phase extracted with CH_2Cl_2 or EtOAc depending on the solubility of the product. The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was removed under reduce pressure. The residue was purified by flash chromatography on silica gel using as eluent *n*-hexane/EtOAc (4:1) to EtOAc (100%) and recrystallized from EtOAc/*n*-hexane to afford the final product (adapted from Ref. 37).

4.1.1.1. 3′,4′-Diphenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1a)[25]. Obtained as a white solid (16.4 mg, 0.0482 mmol, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.95 (br s, 1H), 7.69–7.65 (m, 2H), 7.43–7.27 (m, 3H), 7.28–7.23 (m, 3H, partially obscured by $CHCl_3$ signal), 7.12 (td, J = 7.8, 1.2 Hz, 1H), 7.09–7.05 (m, 2H), 6.80 (d, J = 7.8 Hz, 1H), 6.63 (t, J = 7.8 Hz, 1H), 6.25 (d, J = 7.8 Hz, 1H), 5.08 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 177.08, 159.19, 141.62, 133.68, 130.65, 130.45, 129.15, 129.09, 128.82, 128.58, 128.55, 127.92, 127.40, 123.03, 122.72, 110.21, 89.05, 60.41. Anal. Calcd for $C_{22}H_{16}N_2O_2{\cdot}0.5H_2O$: C, 75.63; H, 4.91; N, 8.02. Found: C, 75.36; H, 4.91; N, 7.82.

4.1.1.2. 3′-(4-Nitrophenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1b). Obtained as a light yellow solid (98.6 mg, 0.256 mmol, 57%). Mp 274–276 °C; IR (KBr): 3198, 1712, 1522, 1337 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ (ppm): 9.58 (br s, 1H), 8.25 (br d, J = 8.8 Hz, 2H), 7.98 (br d, J = 8.8 Hz, 2H), 7.39–7.29 (m, 3H), 7.27–7.21 (m, 2H), 7.10 (t, J = 7.7 Hz, 1H), 6.91 (d, J = 7.7 Hz, 1H), 6.63 (t, J = 7.7 Hz, 1H), 6.29 (d, J = 7.7 Hz, 1H), 5.46 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.54, 158.99, 149.54, 144.31, 135.92, 134.48, 131.69, 130.12, 129.97, 129.45, 129.43, 127.88, 124.86, 123.47, 122.66, 111.02, 90.52, 59.63.

4.1.1.3. 3′-(4-Methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1c). Obtained as a white solid (117.0 mg, 0.316 mmol, 70%). Mp 267–269 °C; IR (KBr): 3198, 1712, 1522, 1337 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ (ppm): 9.51 (br s, 1H), 7.64 (br d, J = 8.9 Hz, 2H), 7.34–7.25 (m, 3H), 7.22–7.18 (m, 2H), 7.15 (t, J = 7.7 Hz, 1H), 6.91 (br d, J = 8.9 Hz, 2H), 6.88 (d, J = 7.7 Hz, 1H), 6.60 (t, J = 7.7 Hz, 1H), 6.24 (d, J = 7.7 Hz, 1H), 5.27 (s, 1H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 177.07, 162.07, 159.43, 144.14, 135.41, 131.21, 130.05, 129.96, 129.63, 128.96, 127.72, 124.18, 122.37, 122.28, 114.96, 110.71, 89.33, 60.49, 55.66; Anal. Calcd for $C_{23}H_{18}N_2O_3{\cdot}0.4H_2O$: C, 73.15; H, 5.03; N, 7.42. Found: C, 72.84; H, 4.90; N, 7.36.

4.1.1.4. 4′-(4-Methoxyphenyl)-3′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1d)[25]. Obtained as a white solid (35.2 mg, 0.0950, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.60 (br s, 1H), 7.70–7.65 (m, 2H, H_{Ar}), 7.38–7.29 (m, 3H), 7.10 (td, J = 7.8, 1.2 Hz, 1H), 6.99 (br d, J = 8.4 Hz, 2H), 6.82–6.76 (m, 3H), 6.66 (td, J = 7.8, 0.8 Hz, 1H), 6.31 (d, J = 7.8 Hz, 1H), 5.03 (s, 1H), 3.76 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 177.64, 159.61, 159.50, 141.84, 130.58, 130.38, 130.24, 128.79, 128.65, 127.91, 127.40, 125.59, 123.07, 122.72, 114.46, 110.46, 89.16, 59.69, 55.36.

4.1.1.5. 3′,4′-Bis(4-methoxyphenyl)-spiro[indoline-3,5′-isoxazoline]-2-one (1f). Obtained as a white solid (114.4 mg, 0.286 mmol, 72%). Mp 219–221 °C; IR (KBr): 3169, 1717, 1260, 1246 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.45 (br s, 1H), 7.64 (br d, J = 9.0 Hz, 2H), 7.16 (td, J = 7.6, 1.2 Hz, 1H), 7.12 (br d, J = 8.7 Hz, 2H), 6.91 (br d, J = 9.0 Hz, 2H), 6.89–6.84 (m, 3H), 6.64 (td, J = 7.6, 1.0 Hz, 1H), 6.32 (d, J = 7.6 Hz, 1H), 5.21 (s, 1H), 3.79 (s, 3H), 3.75 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 177.16, 162.03, 160.45, 159.62, 144.16, 131.20, 131.16, 129.95, 127.89, 127.15, 124.36, 122.41, 122.39, 114.93, 110.67, 89.28, 59.90, 55.66, 55.49; Anal. Calcd for $C_{24}H_{20}N_2O_4 \cdot 0.1C_4H_{10}O$: C, 71.85; H, 5.20; N, 6.87. Found: C, 72.07; H, 5.58; N, 7.14.

4.1.1.6. 5-Nitro-3′,4′-diphenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1g). Obtained as a light yellow solid (74.9 mg, 0.194 mmol, 52%). Mp >320 °C; IR (KBr): 3181, 1726, 1528, 1339 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 10.15 (br s, 1H), 8.17 (dd, J = 8.7, 2.3 Hz, 1H), 7.78–7.73 (m, 2H), 7.48–7.31 (m, 6H), 7.30–7.26 (m, 2H), 7.14 (d, J = 8.7 Hz, 1H), 7.05 (d, J = 2.3 Hz, 1H), 5.51 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.80, 160.01, 150.17, 143.45, 134.39, 131.34, 130.04, 129.86, 129.71, 129.59, 129.45, 128.49, 128.14, 124.87, 123.30, 111.08, 88.67, 60.51; Anal. Calcd for $C_{22}H_{15}N_3O_4 \cdot 0.4H_2O$ C, 67.30; H, 4.06; N, 10.71. Found: C, 67.14; H, 4.13; N, 10.37.

4.1.1.7. 5-Nitro-3′-(4-methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1h). Obtained as a light yellow solid (116.5 mg, 0.280 mmol, 72%). Mp 299–301 °C; IR ν_{max} (KBr): 3188, 1734, 1526, 1337, 1260 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 10.14 (br s, 1H), 8.16 (dd, J = 8.7, 2.4 Hz, 1H), 7.68 (br d, J = 8.9 Hz, 2H), 7.39–7.30 (m, 3H), 7.28–7.24 (m, 2H), 7.13 (d, J = 8.7 Hz, 1H), 7.03 (d, J = 2.4 Hz, 1H), 6.94 (br d, J = 8.9 Hz, 2H), 5.45 (s, 1H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.95, 162.34, 159.51, 150.14, 143.43, 134.62, 130.11, 129.98, 129.87, 129.50, 128.05, 125.08, 123.28, 121.75, 115.10, 111.01, 88.41, 60.79, 55.71; Anal. Calcd for $C_{23}H_{17}N_3O_5 \cdot 0.9H_2O$: C, 64.00; H, 4.40; N, 9.74. Found: C, 63.67; H, 4.01; N, 9.67.

4.1.1.8. 7-Chloro-3′-(4-nitrophenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1i). Obtained as a white solid (76.2 mg, 0.182 mmol, 46%). Mp 240–241 °C; IR (KBr): 3177, 1734, 1518, 1342; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.93 (br s, 1H), 8.26 (br d, J = 8.8 Hz, 2H), 7.98 (br d, J = 8.8 Hz, 2H), 7.38–7.30 (m, 3H), 7.28–7.22 (m, 3H), 6.67 (t, J = 7.8 Hz, 1H), 6.24 (d, J = 7.8 Hz, 1H), 5.54 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.04, 158.97, 149.57, 141.85, 135.59, 134.02, 131.54, 130.06, 130.00, 129.55, 129.43, 126.30, 125.24, 124.83, 123.73, 115.61, 90.72, 59.88.

4.1.1.9. 7-Chloro-3′-(4-methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazolin2]-2-one (1j). Obtained as a white solid (104.6 mg, 0.258 mmol, 66%). Mp 218–219 °C; IR (KBr): 3167, 1724, 1252 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.80 (s, 1H), 7.65 (br d, J = 8.7 Hz, 2H), 7.36–7.27 (m, 3H), 7.26–7.20 (m, 3H), 6.92 (br d, J = 8.7 Hz, 2H), 6.64 (t, J = 7.8 Hz, 1H), 6.19 (d, J = 7.8 Hz, 1H), 5.36 (s, 1H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.59, 162.20, 159.48, 141.74, 135.04, 131.14, 130.06, 130.02, 129.74, 129.13, 126.22, 126.04, 123.51, 122.04, 115.39, 115.02, 89.67, 60.79, 55.68; Anal. Calcd for $C_{23}H_{17}ClN_2O_3 \cdot 1.1H_2O$: C, 65.05; H, 4.52; N, 6.60. Found: C, 65.40; H, 4.68; N, 6.22.

4.1.1.10. 6-Chloro-3′,4′-diphenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1k). Obtained as an off-white solid (77.7 mg, 0.207 mmol, 55%). Mp 271–273 °C; IR (KBr): 3186, 1732 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.67 (s br, 1H), 7.74–7.70 (m, 2H), 7.43–7.27 (m, 6H), 7.25–7.20 (m, 2H), 6.94 (d, J = 2.0 Hz, 1H), 6.65 (dd, J = 8.2, 2.0 Hz, 1H), 6.21 (d, J = 8.2 Hz, 1H), 5.38 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.71, 159.93, 145.58, 136.59, 134.91, 131.10, 129.99, 129.82, 129.75, 129.6, 129.22, 128.95, 128.40, 122.78, 122.29, 111.19, 89.10, 60.24; Anal. Calcd for $C_{22}H_{15}ClN_2O_2$: C, 70.49; H, 4.04; N, 7.48. Found: C, 70.38; H, 4.27; N, 7.25.

4.1.1.11. 6-Chloro-3′-(4-methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1l). Obtained as a white solid (76.9 mg, 0,190 mmol, 49%). Mp 148–150 °C; IR (KBr): 3210, 1748, 1265 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.65 (br s, 1H), 7.64 (br d, J = 8.9 Hz, 2H), 7.36–7.27 (m, 3H), 7.24–7.18 (m, 2H), 6.95–6.88 (m, 3H), 6.64 (dd, J = 8.1, 1.8 Hz, 1H), 6.19 (d, J = 8.1 Hz, 1H), 5.31 (s, 1H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.87, 162.17, 159.45, 145.56, 136.49, 135.14, 130.00, 129.78, 129.14, 128.93, 122.98, 122.24, 122.07, 115.01, 111.13, 88.82, 60.52, 55.67; Anal. Calcd for $C_{23}H_{17}ClN_2O_3 \cdot 0.6H_2O$: C, 66.46; H, 4.42; N, 6.74. Found: C, 66.25; H, 4.34; N, 6.54.

4.1.1.12. 6-Chloro-3′-(4-nitrophenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1m). Obtained as a light yellow solid (70.1 mg, 0.170 mmol, 43%). Mp 255–256 °C; IR (KBr): 3169, 1721, 1522, 1342 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.78 (br s, 1H), 8.26 (br d, J = 9.0 Hz, 2H), 7.98 (br d, J = 9.0 Hz, 2H), 7.39–7.32 (m, 3H), 7.26–7.22 (m, 2H), 6.96 (d, J = 1.9 Hz, 1H), 6.67 (dd, J = 8.2, 1.9 Hz, 1H), 6.25 (d, J = 8.2 Hz, 1H), 5.49 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.32, 158.93, 149.56, 145.67, 136.91, 135.64, 134.13, 130.04, 130.00, 129.55, 129.41, 129.02, 124.83, 122.46, 122.21, 111.39, 89.94, 59.62; Anal. Calcd for $C_{22}H_{14}ClN_3O_4 \cdot 1.0H_2O$: C, 60.35; H, 3.69; N, 9.60. Found: C, 60.13; H, 3.41; N, 9.22.

4.1.1.13. 6-Chloro-4′-phenyl-3′-(*p*-tolyl)-spiro[indoline-3,5′-isoxazoline]-2-one (1n). Obtained as a white solid (107.4 mg, 0.276 mmol, 71%). Mp 136–138 °C; IR (KBr): 3177, 1744 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.65 (br s, 1H), 7.60 (br d, J = 8.2 Hz, 2H), 7.35–7.28 (m, 3H), 7.23–7.16 (m, 4H), 6.93 (d, J = 1.9 Hz, 1H), 6.64 (dd, J = 8.2, 1.9 Hz, 1H), 6.21 (d, J = 8.2 Hz, 1H), 5.34 (s, 1H), 2.30 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.77, 159.80, 145.53, 141.34, 136.53, 135.04, 130.22, 129.99, 129.78, 129.15, 128.94, 128.37, 126.94, 122.89, 122.26, 111.14, 88.94, 60.39, 21.32; Anal. Calcd for $C_{23}H_{17}ClN_2O_2 \cdot 0.3H_2O$: C, 70.06; H, 4.51; N, 7.11. Found: C, 69.79; H, 4.30; N, 6.79.

4.1.1.14. 6-Chloro-3′-(2-methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1o). Obtained as a white solid (121.1 mg, 0.299 mmol, 76%). Mp 128–130 °C; IR (KBr): 3173, 1724, 1248 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.65 (br s, 1H), 7.93 (dd, J = 7.5, 1.7 Hz, 1H), 7.38 (ddd, J = 8.5, 7.5, 1.7 Hz, 1H), 7.25–7.15 (m, 3H), 7.13–7.08 (m, 2H), 7.02 (td, J = 7.5, 0.9 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 6.89 (d, J = 1.9 Hz, 1H), 6.67 (dd, J = 8.1, 1.9 Hz, 1H), 6.47 (d, J = 8.1 Hz, 1H), 5.66 (s, 1H), 3.63 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 177.00, 158.84, 158.40, 145.13, 136.27, 135.31, 132.69, 130.91, 129.90, 129.29, 128.94, 128.60, 123.86, 122.23, 121.62, 118.72, 113.09, 111.10, 89.17, 63.03, 55.85; Anal. Calcd for $C_{23}H_{17}ClN_2O_3 \cdot 0.7H_2O$: C, 66.17; H, 4.45; N, 6.71. Found: C, 66.41; H, 4.73; N, 6.33.

4.1.1.15. 6-Chloro-3′,4′-bis(4-methoxyphenyl)-spiro[indoline-3,5′-isoxazoline]-2-one (1p). Obtained as a white solid (107.0 mg, 0.246 mmol, 70%). Mp 231–232 °C; IR (KBr): 3219, 1748, 1261 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.61 (br s, 1H), 7.63 (br d, J = 8.9 Hz, 2H), 7.12 (br d, J = 8.7 Hz, 2H), 6.95–6.85 (m, 5H), 6.68 (dd, J = 8.1, 1.9 Hz, 1H), 6.28 (d, J = 8.1 Hz, 1H), 5.25 (s, 1H), 3.79 (s, 3H), 3.76 (s, 3H); ^{13}C NMR

(100 MHz, acetone-d_6) δ (ppm): 176.94, 162.12, 160.56, 159.64, 145.54, 136.44, 131.15, 129.99, 129.10, 126.83, 123.15, 122.28, 122.18, 115.08, 114.98, 111.08, 88.79, 59.95, 55.67, 55.53; Anal. Calcd for $C_{23}H_{19}ClN_2O_4$: C, 66.28; H, 4.41; N, 6.44. Found: C, 65.93; H, 4.71; N, 6.28.

4.1.1.16. 6-Bromo-3′,4′-diphenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1q). Obtained as a light yellow solid (61.6 mg, 0.147 mmol, 44%). Mp 288–290 °C; IR (KBr): 3204, 1728 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.68 (br s, 1H), 7.74–7.69 (m, 2H), 7.41–7.36 (m, 3H), 7.36–7.29 (m, 3H), 7.24–7.20 (m, 2H), 7.08 (d, J = 1.7 Hz, 1H), 6.81 (dd, J = 8.0, 1.7 Hz, 1H), 6.15 (d, J = 8.0 Hz, 1H), 5.37 (s, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.62, 159.94, 145.70, 134.89, 131.11, 129.99, 129.83, 129.74, 129.62, 129.23, 128.41, 125.27, 124.70, 123.28, 114.05, 89.17, 60.25; Anal. Calcd for $C_{22}H_{15}BrN_2O_2{\cdot}0.05H_2O$: C, 63.02; H, 3.61; N, 6.68. Found: C, 62.53; H, 3.79; N, 6.61.

4.1.1.17. 6-Bromo-3′-(4-methoxyphenyl)-4′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1r). Obtained as a white solid (109.3 mg, 0.243 mmol, 73%). Mp 247–248 °C; IR (KBr): 3210, 1746, 1265 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ (ppm): 9.67 (br s, 1H), 7.64 (br d, J = 8.9 Hz, 2H), 7.36–7.27 (m, 3H), 7.24–7.18 (m, 2H), 7.08 (d, J = 1.6 Hz, 1H), 6.91 (br d, J = 8.9 Hz, 2H), 6.80 (dd, J = 8.1, 1.7 Hz, 1H), 6.13 (d, J = 8.1 Hz, 1H), 5.32 (s, 1H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ (ppm): 176.78, 162.17, 159.44, 145.66, 135.11, 130.00, 129.77, 129.20, 129.13, 125.21, 124.59, 123.48, 122.05, 115.01, 114.00, 88.90, 60.52, 55.68; Anal. Calcd for $C_{23}H_{17}BrN_2O_3{\cdot}0.6H_2O$: C, 60.04; H, 4.00; N, 6.09. Found: C, 59.73; H, 3.71; N, 5.72.

4.1.1.18. 4′-(4-Methoxyphenyl)-1-methyl-3′-phenyl-spiro[indoline-3,5′-isoxazoline]-2-one (1e). A mixture of 3-(4-methoxybenzylidene)-1-methylindolin-2-one (50.0 mg, 0.188 mmol, 1 equiv), *N*-hydroxybenzimidoyl chloride (88.5 mg, 0.569 mmol, 3 equiv) and zinc (37.2 mg, 0.569 mmol, 3 equiv) in ether (4.7 mL) was stirred at room temperature under nitrogen atmosphere. The solvent was removed under reduced pressure, and the residue was dissolved in EtOAc. After filtered through Celite, the organic solution was washed with brine, dried over anhydrous Na_2SO_4, and the solvent was removed under reduce pressure. The residue was purified by flash chromatography on silica gel using as eluent *n*-hexane/EtOAc (9:1) to EtOAc (100%) and recrystallized from ether to afford the final product 1e as a white solid (18.7 mg, 0.0486 mmol, 22%) (adapted from Ref. 24). Mp 195–196 °C; IR (KBr): 1730, 1250 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.68–7.62 (m, 2H), 7.37–7.27 (m, 3H), 7.22 (t, J = 7.6 Hz, 1H), 6.99 (br d, J = 8.4 Hz, 2H), 6.81–6.74 (m, 3H), 6.71 (t, J = 7.6 Hz, 1H), 6.33 (d, J = 7.6 Hz, 1H), 4.99 (s, 1H), 3.75 (s, 3H), 3.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 175.22, 159.57, 159.54, 144.78, 130.60, 130.31, 130.23, 128.73, 128.69, 127.88, 127.16, 125.69, 122.78, 122.69, 114.43, 108.33, 88.68, 59.59, 55.36, 26.39; Anal. Calcd for $C_{24}H_{20}N_2O_3{\cdot}0.25H_2O$: C, 74.11; H, 5.32; N, 7.20. Found: C, 73.85; H, 5.35; N, 7.12.

4.2. Biological assays

HCT116 and SW620 cells were grown in McCoy's 5A supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA), 1% GlutaMAX™ (Invitrogen) and 1% penicillin/streptomycin solution (Sigma–Aldrich, St Louis, MO, USA), HepG2 human hepatoma cells were grown in DMEM (Invitrogen) supplemented with 10% FBS, 1% non-essential amino acids and 1% antibiotic/antimycotic solution (Sigma–Aldrich) and all cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were seeded at 1×10^5 cells/mL for cell viability assays, and 3×10^5 cells/mL for Western blot and flow cytometry analysis. HCT116 human colorectal carcinoma cells rendered p53-null by somatic knockout[40] was a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD).

4.2.1. Anti-proliferative assays

The cellular growth inhibitory activity was evaluated in four cell lines: human hepatocellular carcinoma cell line [HepG2 (wild-type p53)], an isogenic matched pair of wild type p53 and deleted human colorectal cancer cell lines [HCT116 $p53^{(+/+)}$ and $p53^{(-/-)}$], and human colorectal adenocarcinoma cell lines [SW620 (mut p53)]. Cells were incubated with vehicle or the compounds approximately 24 h after plating. The compounds were dissolved in DMSO and diluted in culture medium to a range of concentrations from 0.5 to 200 μM. Cell viability was assessed 24 h after compound incubation by using the CellTiter96® AQueous Non-Radioactive Cell Proliferation Assay (Promega), using MTS inner salt, according to the manufacturer's protocol. The absorbance was measured at 490 nm using Bio-Rad microplate reader Model 680 (Bio-Rad, Hercules, CA, USA). Nutlin-3 was used as a positive control. The concentrations of the compounds that inhibited cell growth by 50% (GI_{50}) were determined by non-linear regression using GraphPad PRISM software.

4.2.2. Western blot analysis

Total protein extracts from HCT116 $p53^{(-/-)}$ and $p53^{(+/+)}$ cells incubated with vehicle, compounds **1l** or **1n** at 25 and 50 μM for 24 h, were prepared following standard protocols.[41] Protein concentrations were determined using the Bio-Rad protein assay kit, according to the manufacturer's specifications. 60–80 μg of total protein extracts were separated on 8% and 14% (w/v) sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. After electrophoretic transfer onto nitrocellulose membranes, and blocking with a 5% (w/v) non-fat dry milk solution, membranes were incubated overnight at 4 °C with primary mouse monoclonal antibodies reactive to p53 (DO-1), Mdm2 (SMP 14), caspase 3 (H-277) (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal antibody reactive to PARP-1/2 (H-250) (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, a secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (BioRad Laboratories, Hercules, CA, USA) was added for 3 h at room temperature. The membranes were processed for protein detection using the SuperSignal substrate (Pierce Biotechnology, Rockford, IL, USA). β-Actin (AC-15; Sigma–Aldrich) was used as a loading control. The relative intensities of protein bands were analyzed using the QuantityOne Version 4.6 densitometric analysis program (Bio-Rad) and normalized to the corresponding loading control.

4.2.3. Bimolecular fluorescence complementation (BiFC) assay

HCT116 $p53^{(-/-)}$ cells were co-transfected using 1 μg of each BiFC pair plasmid and Lipofectamine 2000 (Invitrogen). 4–6 h after transfection, the medium was replaced with fresh medium and derivatives **1k**, **1l** and **1n** and Nutlin-3 (Sigma–Aldrich) were added (dissolved in DMSO) to a final concentration of 25 and 50 μM. Cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) (Invitrogen Corp.), treated with accutase and harvested with culture medium. Cell suspensions were centrifuged, supernatants discarded, and cell pellets resuspended in PBS. Fluorescence was measured using a FACSCalibur (compound **1k**).

4.3. Stability assays

High-performance liquid chromatography (HPLC) measurements were carried out using a VWR HITACHI assembly equipped with a UV detector L-2400, a column oven L-2300, and a pump L-2130. An injection valve equipped with 20 μL sample loop was used. The separation was performed on a LichroCART® RP-18

(5 μm, 250–4 mm) analytical column (Merck). Acetonitrile/H_2O (70:30, v/v) was used as eluent system. Elution was performed at a solvent flow rate of 1 mL/min. Chromatograms were monitored by UV detection at 260 nm. All analyses were performed at 25 °C. Acquisition and treatment of data were done using Ezchrom Elite software.

4.3.1. Stability in pH 7.4 phosphate buffer

12.5 μL of a 10^{-2} M stock solutions of compounds **1j** and **1r** in DMSO were added to 2.5 mL of potassium phosphate buffer solution (pH 7.4, 0.5 M) at 37 °C. At appropriate intervals, samples (100 μL) were removed and added to acetonitrile (200 μL), and analyzed by HPLC using the methodology previously described. The stability was assessed for a period of 72 h.

4.3.2. Stability in human plasma

Human plasma was obtained from the pooled, heparinized blood of healthy donors, and was frozen and stored at −20 °C prior to use. 12.5 μL of a 10^{-2} M stock solutions of compounds **1j** and **1r** in DMSO were incubated at 37 °C in 2.5 mL of human plasma diluted to 80% (v/v) with potassium phosphate buffer (pH 7.4, 0.5 M). At appropriate intervals, aliquots (100 μL) were removed and added to acetonitrile (200 μL) to quench the reaction and precipitate plasma proteins. These samples were vortexed, centrifuged and the supernatant was analyzed by HPLC using the methodology previously described. The stability was assessed for a period of 72 h.

4.3.3. Stability in rat microssomes

Male Rat Pooled Liver Microsomes (Sprague–Dawley) BD Gentest™ were used. A mixture of 282 μL purified water, 88 μL potassium phosphate (pH 7.4, 0.5 M), 20 μL NADPH Regenerating System Solution A (BD Biosciences Cat. No. 451220), 4 μL NADPH Regenerating System Solution B (BD Biosciences Cat. No. 451200), and 10 μL of microsomes was incubated 5 min at 37 °C in a water bath before addition of 4 μL of a 10^{-3} M stock solution of the tested compounds (**1j** and **1r**) in DMSO. At appropriate intervals, aliquots (45 μL) were removed and added to acetonitrile (45 μL). These samples were mixed, centrifuged and the supernatant was analyzed by HPLC using the methodology previously described. The half-life was determined applying the pseudo-first-order reaction equation: $t_{1/2} = \ln(2)/k$.

The viability of the rat microsomes was verified by evaluating their CYP2E1-catalyzed *p*-nitrophenol hydroxylation capacity, applying a methodology described elsewhere.[42]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.048.

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