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Research paper

Spirotriazoline oxindoles: A novel chemical scaffold with *in vitro* anticancer properties

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ABSTRACT

The design and synthesis of a library of twenty-six spirotriazoline oxindoles and their *in vitro* evaluation as potential anticancer agents is reported. The antiproliferative activity of the synthesized compounds was assessed against four different cancer cell lines (HCT-116 *p53* $^{(+/+)}$, HCT-116 *p53* $^{(-/-)}$, MCF-7, and MDA-MB-231). Four spirotriazoline oxindoles showed selectivity against the four cancer cell lines tested over the non-cancer derived HEK 293T cell line. To characterize the molecular mechanisms involved in compound antitumoral activity, two spirotriazoline oxindoles were selected for further studies. Both compounds were able to induce apoptosis and cell cycle arrest at G0/G1 phase and upregulated p53 steady-state levels, while decreasing its main inhibitor MDM2, in HCT-116 cells. Importantly, cytotoxic effects induced by spirotriazoline oxindoles occurred in cancer cells without eliciting cell death in non-malignant CCD-18Co human colon fibroblasts. In addition, four spirotriazoline oxindoles showed selectivity against the triple-negative breast cancer cell line MDA-MB-231 with IC_{50} values of 3.5–6.7 μM. These results highlight the anticancer potential of spirotriazoline oxindoles, especially when dealing with aggressive and challenging triple-negative breast cancer.

1. Introduction

It is well established that cancer represents a major public health issue worldwide. In 2012, it was estimated 14.1 million new cancer cases and 8.2 million deaths [1], and the World Health Organization estimates a rise of 13.1 million cancer deaths until 2030. Specifically, breast cancer is the most common cancer in woman, and is the leading cause of cancer death in women aged 20–59 years in the USA [2]. In particular, a specific tumor subtype, known as triple-negative breast cancer (TNBC) is characterized as a more aggressive type of cancer with poorer prognostic. TNBCs are characterized by the lack of expression of estrogen and progesterone receptors, and absence of human epidermal growth factor receptor 2 overexpression, representing the hardest breast cancer to treat [3]. Although early detection and efficient therapies contributed to a decrease of cancer mortality in developed countries, overcoming drug intrinsic and acquired resistance represents a major challenge [4,5] and imposes a constant development of new molecules and the identification of novel targets, especially for TNBC patients [3].

Heterocycles possessing a spirooxindole framework are found in many natural products and medicinal agents with diverse biological activities [6–8]. The attractiveness of this type of scaffold in organic and medicinal chemistry is evident by the increasing number of publications on this topic over the last 10 years. This can be ascribed, at least partially, to the fact that the central spiro carbon atom imposes a conformational restriction to the structure that can be beneficial for ligand-target binding, and thus potentially promoting an increase in potency and/or specificity [7].

Furthermore, the reduced molecular flexibility displayed by the spirooxindole scaffold can also potentially lead to better pharmacokinetic properties [9]. Antiproliferative activity against cancer cell lines is one of the biological effects reported for spirooxindoles (Fig. 1) [10]. Specifically, CFI-400945 is a Polo-like kinase 4 (PLK4) inhibitor [11], compound (−)-**1** interferes with microtubule polymerization and arrests mitosis [12], and compounds MI-77301 [13] and SM13 [14] modulate p53 activity.

We have recently developed several small molecules showing potential anticancer activity [15–23], with a focus on novel five-membered ring spirooxindoles that act as p53 modulators (Fig. 2). Initial studies explored the spiroisoxazoline oxindole

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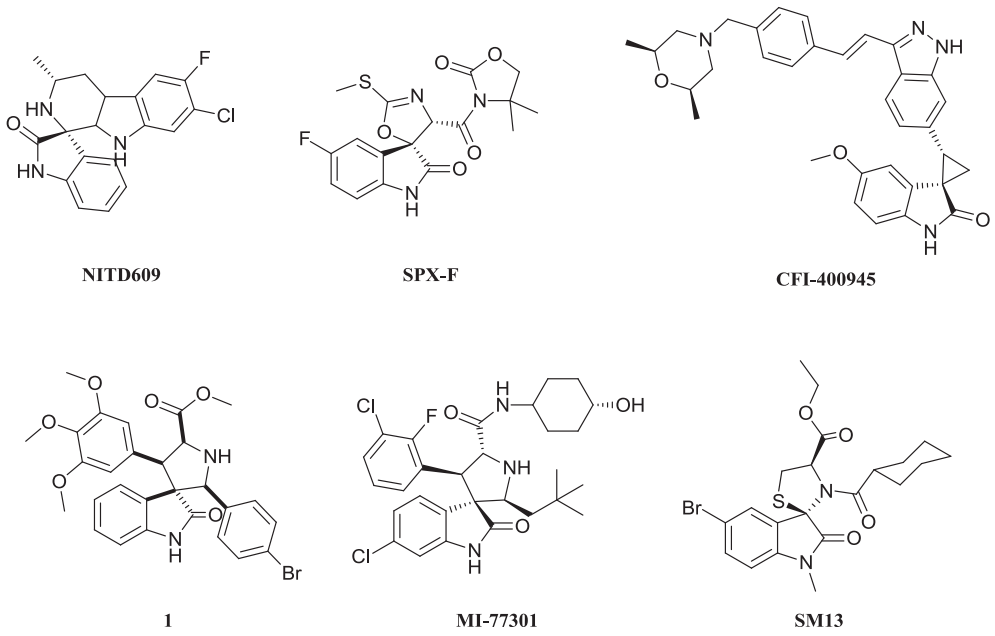


Fig. 1. Spirooxindoles with biological activity.

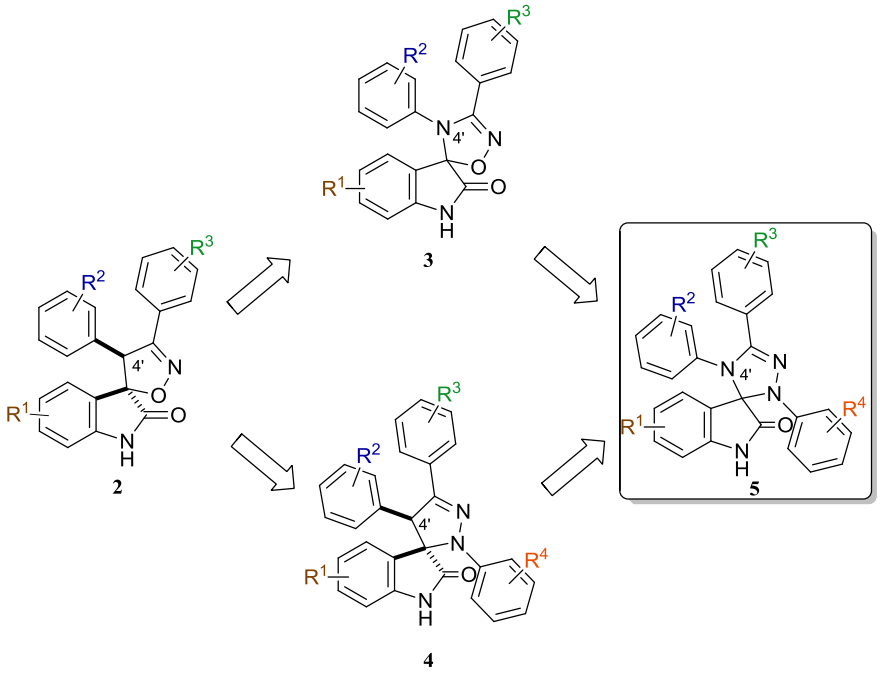
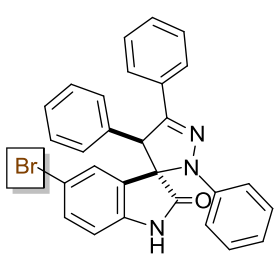


Fig. 2. Optimization strategy towards spirotriazoline oxindoles **5**.

scaffold **2** [21]. Then, the spirooxadiazoline oxindole scaffold **3** was obtained by replacing the chiral isoxazoline C4′ for a N4′ atom. Further optimization led to a spirooxadiazoline oxindole 15.6-fold more potent in HCT-116 colorectal cancer cells than the most active spiroisoxazoline oxindole previously developed by us [15]. We also explored the addition of a fourth lipophilic moiety to the main scaffold, by changing the isoxazoline oxygen by a *N*-phenyl group (spiropyrazoline oxindole scaffold **4**) [20,23]. These compounds were tested in two breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231. One compound, spiropyrazoline oxindole **4a**, was found to be at least 13.7-fold more potent against MCF-7 cells over MDA-MB-231 cells (TNBC cell line), and the non-cancer derived human embryonic kidney HEK 293T cell line (Fig. 3).

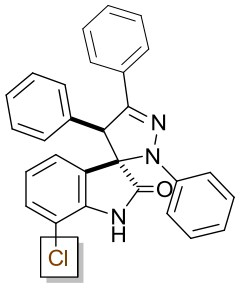
Interestingly, spiropyrazoline oxindole **4b** showed approximately the same potency against both breast cancer cell lines, but maintained selectivity (>4.5-fold) over the HEK 293T cell line. This observation suggests that it would be possible to obtain differential selectivity in breast cancer cell lines depending on the position of the halogen in the oxindole moiety.

Since both strategies gave rise to improved and/or more selective compounds, we decided to apply and explore simultaneously both optimization strategies: replacing the chiral isoxazoline C4′



4a

MCF-7 IC_{50}= 7.3 μM
MDA-MB-231 IC_{50}> 100 μM
HEK 293T IC_{50}> 100 μM



4b

MCF-7 IC_{50}= 22.4 μM
MDA-MB-231 IC_{50}= 16.9 μM
HEK 293T IC_{50}> 100 μM

Fig. 3. Chemical structure of spiropyrazoline oxindoles **4a** and **4b**.

for a N4′ atom, and changing the isoxazoline oxygen by a *N*-phenyl group (Fig. 2). As a result, a series of spirotriazoline oxindoles **5** was synthesized and their anticancer activity was evaluated. These new five-membered ring spirooxindoles resulted from the juxtaposition of oxindole and 1,2,4-triazole moieties, two scaffolds that are described to have anticancer activity [24–27]. We started by performing a screening in breast cancer cell lines MCF-7 and MDA-MB-231. For the most active compounds we evaluated their activity in non-cancer HEK 293T cells. To amplify their anticancer scope, and compare with our previous results obtained with related five-membered ring spirooxindoles, we further assessed their antiproliferative activity in two additional cancer cell lines: the HCT116 human colorectal carcinoma isogenic pair, with and without p53. The mechanism of action of the most promising compounds was further evaluated.

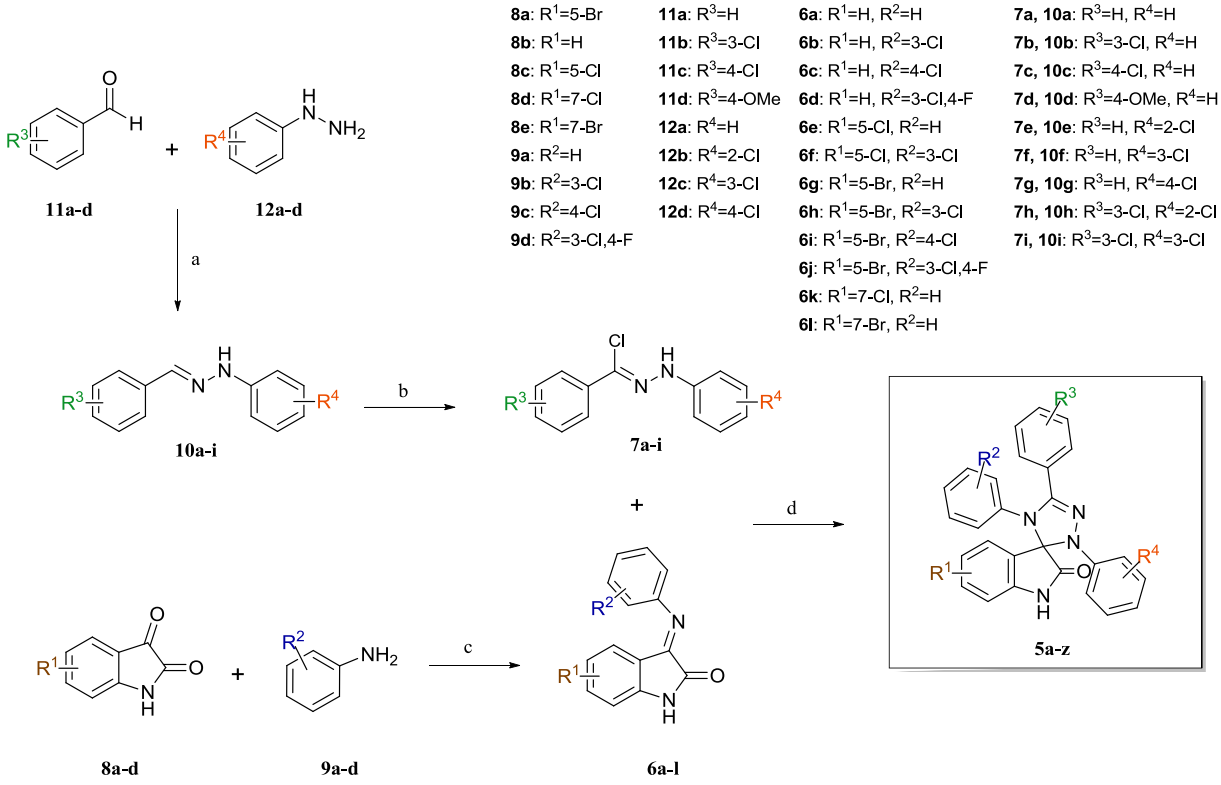
2. Results and discussion

2.1. Synthesis of compounds

Spirotriazoline oxindoles **5a-z** were synthesized by 1,3-dipolar cycloaddition between 3-arylimino indolin-2-ones **6** and nitrile imines (Scheme 1, Table 1). The 1,3-dipoles were generated *in situ* by dehydrohalogenation of hydrazonoyl chlorides **7**, mediated by triethylamine (60–95% yield) [28,29]. 3-Arylimino indolin-2-ones **6** were synthesized by reacting indoline-2,3-diones (**8**) with different anilines (**9**), in the presence of acetic acid (68–93% yield) [15]. The hydrazonoyl chlorides **7** were synthesized by reacting *N*-chlorosuccinimide-dimethyl sulphide complex prepared *in situ*, with the appropriate *N*-arylhydrazone (**10**) at −78 °C (62–88% yield) [20]. Hydrazones (**10**) were prepared by reacting benzaldehyde derivatives (**11**) with phenylhydrazines (**12**) in aqueous ethanol 20% (81–99% yield) [30].

The spiropyrazoline equivalent (compound **4c**) of the most potent compound in MDA-MB-231 breast cancer cell line (compound **5i**) was also synthesized (Scheme 2). Spiropyrazoline oxindole **4c** was obtained in 65% yield by reacting 3-methylene indoline-2-one **13** with hydrazonoyl chloride **7a** (Scheme 2). Compound **13** was synthesized by aldolic condensation between 5-bromoindolin-2-one (**14**) and 3-chlorobenzaldehyde [21], and the former by reduction of 5-bromoindolin-2,3-dione (**8a**) in the presence of $TiCl_4$/Zn [31], as described in literature.

All compounds gave spectroscopic data in agreement with their structures. For all spirotriazolines, the regioisomer formed was the spiro[indoline-3,3′-triazoline]-2-one as shown by the chemical shift of the spiro carbon signal at 87.88–89.83 ppm, in line with reported data [28]. The regioisomer formed for the spiropyrazoline oxindole **4c** was established to be the spiro[indoline-3,3′-pyrazoline]-2-one [20,32]: the chemical shift of the spiro carbon appears at 77.22 ppm, the H-4′ at 5.45 ppm, and C-4′ at 62.34 ppm. The



8a: R^1=5-Br
8b: R^1=H
8c: R^1=5-Cl
8d: R^1=7-Cl
8e: R^1=7-Br
9a: R^2=H
9b: R^2=3-Cl
9c: R^2=4-Cl
9d: R^2=3-Cl,4-F

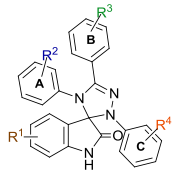
11a: R^3=H
11b: R^3=3-Cl
11c: R^3=4-Cl
11d: R^3=4-OMe
12a: R^4=H
12b: R^4=2-Cl
12c: R^4=3-Cl
12d: R^4=4-Cl

6a: R^1=H, R^2=H
6b: R^1=H, R^2=3-Cl
6c: R^1=H, R^2=4-Cl
6d: R^1=H, R^2=3-Cl,4-F
6e: R^1=5-Cl, R^2=H
6f: R^1=5-Cl, R^2=3-Cl
6g: R^1=5-Br, R^2=H
6h: R^1=5-Br, R^2=3-Cl
6i: R^1=5-Br, R^2=4-Cl
6j: R^1=5-Br, R^2=3-Cl,4-F
6k: R^1=7-Cl, R^2=H
6l: R^1=7-Br, R^2=H

7a, 10a: R^3=H, R^4=H
7b, 10b: R^3=3-Cl, R^4=H
7c, 10c: R^3=4-Cl, R^4=H
7d, 10d: R^3=4-OMe, R^4=H
7e, 10e: R^3=H, R^4=2-Cl
7f, 10f: R^3=H, R^4=3-Cl
7g, 10g: R^3=H, R^4=4-Cl
7h, 10h: R^3=3-Cl, R^4=2-Cl
7i, 10i: R^3=3-Cl, R^4=3-Cl

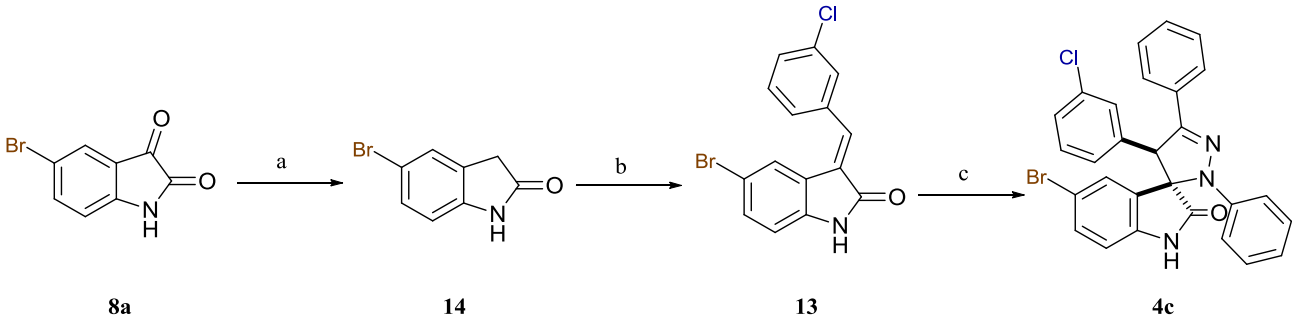
Scheme 1. Synthesis of spirotriazoline oxindoles **5a-z**: (a) aqueous ethanol 20%, r.t., 2–3 h, 81–99%; (b) NCS, $S(CH_3)_2$, 0 °C, 15 min; −78 °C, 1 h, then allowed to warm up to r.t., 62–88%.; (c) CH_3COOH, EtOH, reflux, 3–24 h, 68–93%; (d) Et_3N, DCM, r.t., 24 h, 60–95%.

Table 1
In vitro antiproliferative activities of spirotriazoline oxindoles **5a-z** toward breast cancer cell lines and non-tumor cell line.



Compound	R^1	R^2	R^3	R^4	Breast cancer cell lines		Non-cancer cell line	SI[b,d]	SI[c,d]
					MCF-7 IC_{50}, μM[a]	MDA-MB-231 IC_{50}, μM[a]	HEK 293T IC_{50}, μM[a]		
5a	H	H	H	H	36.2 ± 3.3	23.4 ± 4.3	ND		
5b	H	4-Cl	H	H	24.1 ± 4.8	10.7 ± 1.0	>100	2.3	>4.1
5c	H	3-Cl	H	H	13.7 ± 0.4	12.5 ± 1.1	25.7 ± 5.6		
5d	H	3-Cl,4-F	H	H	19.5 ± 0.7	32.3 ± 4.0	ND		
5e	5-Cl	H	H	H	19.1 ± 0.8	11.7 ± 0.6	32.6 ± 9.3		
5f	5-Cl	3-Cl	H	H	9.8 ± 1.5	8.9 ± 1.9	>100		>10.2
5g	5-Br	H	H	H	23.0 ± 1.7	6.7 ± 0.4	56.3 ± 11.3	3.4	2.4
5h	5-Br	4-Cl	H	H	16.8 ± 1.0	5.2 ± 1.0	21.6 ± 6.8	3.2	
5i	5-Br	3-Cl	H	H	21.4 ± 2.2	3.5 ± 1.0	21.6 ± 6.8	6.1	
5j	5-Br	3-Cl, 4-F	H	H	11.0 ± 2.4	4.6 ± 0.3	16.6 ± 3.7	2.4	
5k	7-Cl	H	H	H	10.8 ± 0.9	8.9 ± 0.6	>100		>9.3
5l	7-Br	H	H	H	21.8 ± 1.6	23.7 ± 4.5	ND		
5m	H	4-Cl	3-Cl	H	13.1 ± 2.4	11.6 ± 1.3	20.5 ± 5.3		
5n	H	4-Cl	4-Cl	H	10.9 ± 2.0	8.2 ± 0.8	22.7 ± 5.3		2.1
5o	H	4-Cl	H	2-Cl	28.4 ± 5.0	24.8 ± 0.8	ND		
5p	H	4-Cl	H	3-Cl	14.2 ± 2.4	15.7 ± 2.4	15.4 ± 2.6		
5q	H	4-Cl	H	4-Cl	10.1 ± 0.5	9.0 ± 1.2	15.8 ± 4.4		
5r	H	4-Cl	3-Cl	2-Cl	13.1 ± 0.5	14.5 ± 0.5	35.0 ± 12.2		2.7
5s	5-Br	3-Cl	3-Cl	H	8.0 ± 1.4	10.2 ± 1.3	12.5 ± 1.1		
5t	5-Br	3-Cl	4-Cl	H	10.1 ± 0.9	14.6 ± 2.1	20.3 ± 6.5		
5u	5-Br	3-Cl	4-OMe	H	12.3 ± 1.5	14.1 ± 1.1	25.9 ± 5.8		
5v	5-Br	3-Cl	H	2-Cl	10.2 ± 0.8	11.7 ± 1.7	20.9 ± 3.0		
5w	5-Br	3-Cl	H	3-Cl	9.8 ± 0.9	10.7 ± 3.1	16.9 ± 3.4		
5x	5-Br	3-Cl	H	4-Cl	11.4 ± 0.9	15.0 ± 4.4	16.2 ± 2.4		
5y	5-Br	3-Cl	3-Cl	2-Cl	9.5 ± 3.1	7.6 ± 2.4	21.4 ± 7.4		2.3
5z	5-Br	3-Cl	3-Cl	3-Cl	6.9 ± 0.7	7.0 ± 1.9	9.0 ± 1.7		

ND: not determined.
[a] IC_{50} determined by the MTT method after 72 h. Each value is the mean (IC_{50} ± SD) of three independent experiments.
[b] Selectivity index against MDA-MB-231 expressed by the ratio MDA-MB-231 IC_{50}/MCF-7 IC_{50}.
[c] Selectivity index towards cancer cell lines over HEK 293T, expressed by the ratio HEK 293T IC_{50}/highest IC_{50} obtained in breast cancer cell lines.
[d] Selectivity is depicted when SI > 2.



Scheme 2. Synthesis of spiropyrazoline oxindole **4c**: (a) $TiCl_4$, Zn, THF, 65%; (b) 3-chlorobenzaldehyde, piperidine, EtOH, reflux, 5 h, 94%; (c) **7a**, Et_3N, DCM, r.t., 24 h, 75%.

relative stereochemistry of spirooxindole **4c** was assigned by comparison with reported spiropyrazoline oxindole X-ray crystallography structure [33].

2.2. Biological evaluation

2.2.1. Structure-activity relationship studies (SAR)

The SAR study for spirotriazoline oxindoles **5** was performed primarily in human breast cancer cell lines (MCF-7 and MDA-MB-231) for better comparison with the structurally similar spiropyrazoline oxindoles **4** previously reported. Since colorectal cancer (CRC) is one of the most commonly diagnosed cancers and presents the highest cause of cancer deaths [1], the scope of anticancer activity of all spirotriazoline oxindoles with a IC_{50} lower than 15 μM in MCF-7 cells was further expanded to p53 wild-type and null isogenic (*p53* $^{(+/+)}$ and *p53* $^{(-/-)}$) HCT-116 colorectal cancer cell line. Specifically, we were interested in evaluating if these compounds could have an effect mediated by a p53-dependent pathway. These compounds were also tested in the non-cancer derived HEK 293T to assess their cytotoxicity to normal cells. The IC_{50} values obtained in the five cell lines are presented on Tables 1 and 2.

We started our study by synthesizing and evaluating

spirotriazoline oxindoles **5e**, **5g**, and **5k-l** with the same substituents of spiropyrazoline oxindoles **4a** and **4b** (chlorine and bromine at position 5 or 7 of the oxindole and no substituents in the phenyl rings), and the non-halogenated counterpart **5a**. In this initial study, the comparison between antiproliferative activities with their spiropyrazoline oxindole counterparts (**5a**, **5e**, **5g** and **5k** versus **4a**-**b**, and **4d**-**e**) revealed that in breast cancer cell lines (Table 3):

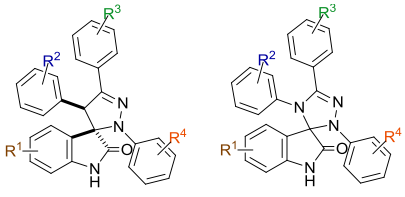
- all spirotriazoline oxindoles were more active against the MCF-7 cell line, with the exception of compound **5g**, containing a bromine atom at C-5 indole (IC_{50} = 23.0 μM vs. **4a**, IC_{50} = 7.3 μM). However, spirotriazoline oxindole **5g** was at least 14.9-fold more potent against MDA-MB-231 cell line, revealing that by altering C4′ for N4′ it is possible to change selectivity between breast cancer cell lines, going from a compound at least 13.7-fold more selective towards MCF-7 cells (**4e**) to a derivative 3.4-fold more selective toward MDA-MB-231 cells (**5g**).

Table 2
In vitro antiproliferative activities of spirotriazoline oxindoles **5a-z** toward colorectal cancer cell lines and non-cancer cell line.

Compound	R^1	R^2	R^3	R^4	Colorectal cancer cell lines		Non-cancer cell line	SI[c,d]
					HCT-116 $p53^{(+/+)}$ IC_{50}, μM[a]	HCT-116 $p53^{(-/-)}$ IC_{50}, μM[a]	HEK 293T IC_{50}, μM[b]	
5a	H	H	H	H	ND	ND	ND	
5b	H	4-Cl	H	H	15.0 ± 1.5	18.5 ± 1.2	>100	>4.1
5c	H	3-Cl	H	H	19.9 ± 1.4	22.1 ± 2.0	25.7 ± 5.6	
5d	H	3-Cl,4-F	H	H	ND	ND	ND	
5e	5-Cl	H	H	H	21.6 ± 0.6	22.1 ± 3.2	32.6 ± 9.3	
5f	5-Cl	3-Cl	H	H	16.3 ± 0.2	17.9 ± 0.6	>100	>5.6
5g	5-Br	H	H	H	22.2 ± 0.5	22.5 ± 4.4	56.3 ± 11.3	2.5
5h	5-Br	4-Cl	H	H	16.3 ± 1.2	18.2 ± 0.9	21.6 ± 6.8	
5i	5-Br	3-Cl	H	H	15.7 ± 1.4	17.8 ± 2.0	21.6 ± 6.8	
5j	5-Br	3-Cl, 4-F	H	H	13.5 ± 1.0	15.9 ± 1.3	16.6 ± 3.7	
5k	7-Cl	H	H	H	23.7 ± 0.6	20.1 ± 1.7	>100	>4.2
5l	7-Br	H	H	H	ND	ND	ND	
5m	H	4-Cl	3-Cl	H	17.4 ± 2.4	16.1 ± 1.6	20.5 ± 5.3	
5n	H	4-Cl	4-Cl	H	16.8 ± 2.2	18.5 ± 1.6	22.7 ± 5.3	
5o	H	4-Cl	H	2-Cl	ND	ND	ND	
5p	H	4-Cl	H	3-Cl	19.8 ± 1.4	18.5 ± 0.9	15.4 ± 2.6	
5q	H	4-Cl	H	4-Cl	18.5 ± 0.9	18.7 ± 0.5	15.8 ± 4.4	
5r	H	4-Cl	3-Cl	2-Cl	25.3 ± 1.3	34.6 ± 2.9	35.0 ± 12.2	
5s	5-Br	3-Cl	3-Cl	H	12.6 ± 1.2	13.0 ± 1.4	12.5 ± 1.1	
5t	5-Br	3-Cl	4-Cl	H	10.8 ± 1.2	10.8 ± 0.9	20.3 ± 6.5	
5u	5-Br	3-Cl	4-OMe	H	22.2 ± 1.4	25.8 ± 1.2	25.9 ± 5.8	
5v	5-Br	3-Cl	H	2-Cl	21.5 ± 2.1	31.6 ± 1.5	20.9 ± 3.0	
5w	5-Br	3-Cl	H	3-Cl	12.0 ± 1.1	13.1 ± 1.4	16.9 ± 3.4	
5x	5-Br	3-Cl	H	4-Cl	15.0 ± 1.3	17.2 ± 1.8	16.2 ± 2.4	
5y	5-Br	3-Cl	3-Cl	2-Cl	13.9 ± **1.1**	17.7 ± **1.3**	21.4 ± 7.4	
5z	5-Br	3-Cl	3-Cl	3-Cl	10.2 ± 1.7	9.0 ± 0.8	9.0 ± 1.7	
Nutlin-3a	–	–	–	–	4.0 ± 1.2	47.8 ± 1.9	–	–

ND: not determined.
[a] IC_{50} determined by the MTS method after 72 h. Each value is the mean (IC_{50} ± SD) of three independent experiments performed in duplicate.
[b] IC_{50} determined by the MTT method after 72 h. Each value is the mean (IC_{50} ± SD) of three independent experiments.
[c] Selectivity index towards cancer cell lines over HEK 293T, expressed by the ratio HEK 293T IC_{50}/highest IC_{50} obtained in cancer cell lines.
[d] Selectivity is depicted when SI > 2.

Table 3
In vitro antiproliferative activities of spiropyrazoline oxindoles **4** *vs* spirotriazoline oxindoles **5**.



4 **5**

R^1	R^2	R^3	R^4	Compd	MCF-7 IC_{50}, μM	MDA-MB-231 IC_{50}, μM	Compd	MCF-7 IC_{50}, μM	MDA-MB-231 IC_{50}, μM
5-Br	H	H	H	**4a**	7.3 ± 1.4	>100	**5g**	23.0 ± 1.7	6.7 ± 0.4
7-Cl	H	H	H	**4b**	22.4 ± 3.5	16.9 ± 1.2	**5k**	10.8 ± 0.9	8.9 ± 0.6
5-Br	3-Cl	H	H	**4c**	10.0 ± 0.7	12.6 ± 0.3	**5i**	21.4 ± 2.2	3.5 ± 1.0
H	H	H	H	**4d**	>100	ND	**5a**	36.2 ± 3.3	23.4 ± 4.3
5-Cl	H	H	H	**4e**	37.7 ± 14.1	ND	**5e**	19.1 ± 0.8	11.7 ± 0.6

IC_{50} determined by the MTT method after 72 h. Each value is the mean (IC_{50} ± SD) of three independent experiments.
ND: not determined.

- the non-halogenated spirotriazoline oxindole (**5a**) was at least 2.8-fold more potent against the MCF-7 cell line than the spiropyrazoline oxindole (**4d**) counterpart (**5a**, IC_{50} = 36.2 μM vs. **4d**, IC_{50} > 100 μM).
- 5-chlorooxindole spirotriazoline oxindole (**5e**) was 2.0-fold more potent in the MCF-7 cell line than its spiropyrazoline (**4e**) counterpart (**5e**, IC_{50} = 19.1 μM vs. **4e**, IC_{50} = 37.7 μM).
- 7-chlorooxindole spirotriazoline oxindole (**5k**) was also 2-fold more potent against both breast cancer cell lines (MCF-7: IC_{50} = 10.8 μM vs. **4b**, IC_{50} = 22.4 μM; MDA-MB-231: IC_{50} = 8.9 μM vs. **4b**, IC_{50} = 16.9 μM).

In the spirotriazoline series, a 2.0-fold decrease in potency in both breast cancer cells lines was observed when 7-chlorine (**5k**) was changed to 7-bromine (MCF-7: **5l**, IC_{50} = 21.8 μM vs. **5k**, IC_{50} = 10.8 μM; MDA-MB-231: **5l**, IC_{50} = 23.7 μM vs. **5k**, IC_{50} = 8.9 μM).

The remarkable selectivity of compound **5g** towards the TNBC cell line MDA-MB-231 (IC_{50} of 6.7 μM) spurred the synthesis of new derivatives with 5-bromoxindole moiety. We started by probing ring A at *meta* and *para* position (**5h-j**). In general, any additional halogen to one or both positions tested led to an activity improvement in MCF-7 cells, reaching a 2.1-fold increase in potency for compound **5j**. Interestingly, comparing these 5-bromooxindole derivatives to their non-halogenated oxindole counterparts (**5b-d**) revealed that their activities were fairly similar (less than 1.8-fold difference) in MCF-7 cells. However, in the MDA-MB-231 cell line, 5-bromooxindole derivatives were substantially more active, with derivative **5j** (R^2 = 3-Cl, 4-F) showing a 7.0-fold increase in potency (**5j**, IC_{50} = 4.6 μM vs. **5d**, IC_{50} = 32.3 μM). The most active spirotriazoline oxindole in MDA-MB-231 cells was compound **5i** (R^1 = 5-Br and R^2 = 3-Cl) with a IC_{50} of 3.5 μM, representing a 1.9-fold activity improvement and better selectivity between breast cancer cell lines in comparison to the non-halogenated ring A counterpart **5g**. Furthermore, maintaining the same substituent in ring A (R^2 = 3-Cl), but changing the halogen at R^1 from 5-bromo to 5-chloro, the selectivity between breast cancer cell lines was lost (**5f** vs. **5i**).

Then we decided to investigate different substitutions at rings B and C (spirotriazoline oxindoles **5m-z**, Table 1). Two compounds were selected as starting points for this endeavor: the most active compound in the MDA-MB-231 cells (compound **5i**), and the non-toxic derivative (HEK-293T IC_{50} > 100 μM) with selectivity between the two breast cancer cell lines (compound **5b**). Except for compound **5o**, introducing chlorine atoms in different positions in one or both rings doubled the potency in MCF-7 cells (compounds **5m-n**, **5p-t**, and **5v-z**). The most active spirotriazoline oxindole tested in MCF-7 cells was compound **5z** (MCF-7 IC_{50} = 6.9 μM). However, these new substitutions did not improve activity in the MDA-MB-231 cell line. In fact, for all 5-bromooxindole derivatives at least a 2-fold decrease in activity was observed. The same pattern of response was also achieved for compound **5u** (R^3 = 4-OMe). Interestingly, three compounds still showed 2.0-fold higher selectivity towards both breast cancer cell lines over HEK 293K cells, making a total of seven selective derivatives (**5b**, **5f-g**, **5k**, **5n**, **5r**, **5y**). In addition, derivative **5r** also showed a 2.0-fold selectivity over both HCT116 cell lines.

More importantly, from this study performed in four cancer cell lines and HEK 293T, we were able to achieve four derivatives (**5g-j**) selective against MDA-MB-231 cells (≥2.4-fold) over the other four cell lines. In addition, four derivatives (**5b**, **5f-g**, **5k**) showed selectivity towards all four cancer cell lines (≥2.5-fold) over the non-cancer derived cell line.

The spiropyrazoline oxindole **4c**, with the same substitution pattern as the most potent compound in MDA-MB-231 cancer cell line (compound **5i**), was found to be 3.6-fold less active in MDA-MB-231 cells, and 2.2-fold more potent in MCF-7 cells than compound **5i**. This compound lost selectivity among breast cancer cell lines, and the non-cancer cell line HEK 293T (HEK 293T IC_{50} = 17.5 μM).

2.2.2. Study of cell death mechanism

To further explore the molecular mechanisms underlying compound-mediated loss of cell viability, we selected compounds with different and promising antiproliferative profiles: (a) spirotriazoline oxindole **5f**, the most selective compound against all four cancer cell lines over HEK 293T cells; (b) spirotriazoline oxindole **5g,** the most selective derivative toward MDA-MB-231 breast cancer cell line; and (c) spirotriazoline oxindole **5y**, the most active compound in the HCT116 $p53^{(+/+)}$ colon cancer cell line with at least a 1.3-fold more potency over HCT116 $p53^{(-/-)}$ cells to assess the involvement of p53-MDM2 interaction, as observed with our previous spirooxindoles.

We evaluated apoptosis and cell cycle progression after treatment of cells with spirotriazoline oxindoles **5f** and **5y**, by flow cytometry analysis, using the HCT-116 $p53^{(+/+)}$ cancer cell line. At the IC_{50} concentration, both compounds were able to induce apoptosis in HCT-116 $p53^{(+/+)}$ cells, as observed by the significant increase of 3.1-fold ($p < 0.01$) and 2.8-fold ($p < 0.05$), respectively, in the percentage of cells in early apoptosis (Fig. 4A). Furthermore, a robust dose-dependent response was observed when a 2xIC_{50} concentration was used. In addition, after 24 h of exposure, compound **5f** induced a G0/G1 phase arrest, as detected by a 50% increase of cells in this phase, while promoting a decrease of approximately 10% in the G2/M phase (Fig. 4B). Spirotriazoline oxindole **5y** was also able to promote a 22% decrease of cells in G2/M phase.

After 48 h, approximately 54% of cells treated with both compounds **5f** and **5y** were in the G0/G1 phase, whereas only 42% of the control cells progressed into this phase. Moreover, compounds **5f** and **5y** decreased the percentage of cells in G2/M phase in about 15% and 10%, respectively.

Regarding the mode of action of compound **5g** in MDA-MB-231 breast cancer cells, we detected at the IC_{50} concentration a 2.5-fold increase in caspase-3 and -7 activity, comparing with DMSO (vehicle control) and Doxorubicin (positive control) (Fig. 5), indicative of apoptosis. However, the presence of **5g** did not affect MDA-MB-231 cell cycle progression (data not shown).

Spirotriazoline oxindole **5y** only displayed a 1.5-fold selectivity index between HCT-116 $p53^{(+/+)}$ colon cancer cells and HEK 293T cells. Therefore, to reassure their non-cytotoxic effect to non-cancer derived cells at tested concentrations (IC_{50}), we further evaluated their growth inhibition potential in CCD-18Co human normal colon fibroblasts using the IC_{50} and IC_{80} equitoxic concentrations, previously determined for HCT-116 $p53^{(+/+)}$ cells. As observed in Fig. 6, treatment with compounds **5f** and **5y** at the IC_{50} concentration did not affect CCD-18Co cell viability. When we used higher concentrations, we could detect a reduction of approximately 40% in cell viability, which is still far below the values obtained for the HCT-116 $p53^{(+/+)}$ cell line. The absence of cytotoxic effects induced by compounds **5f** and **5y** in human normal colon fibroblasts is clearly an advantage for using these compounds as potential therapeutic agents.

Since our previous spirooxindoles studied were able to disrupt the interaction between p53 and MDM2 [15,21], we also explored the effect of spirotriazoline oxindole **5y** on this interaction. By

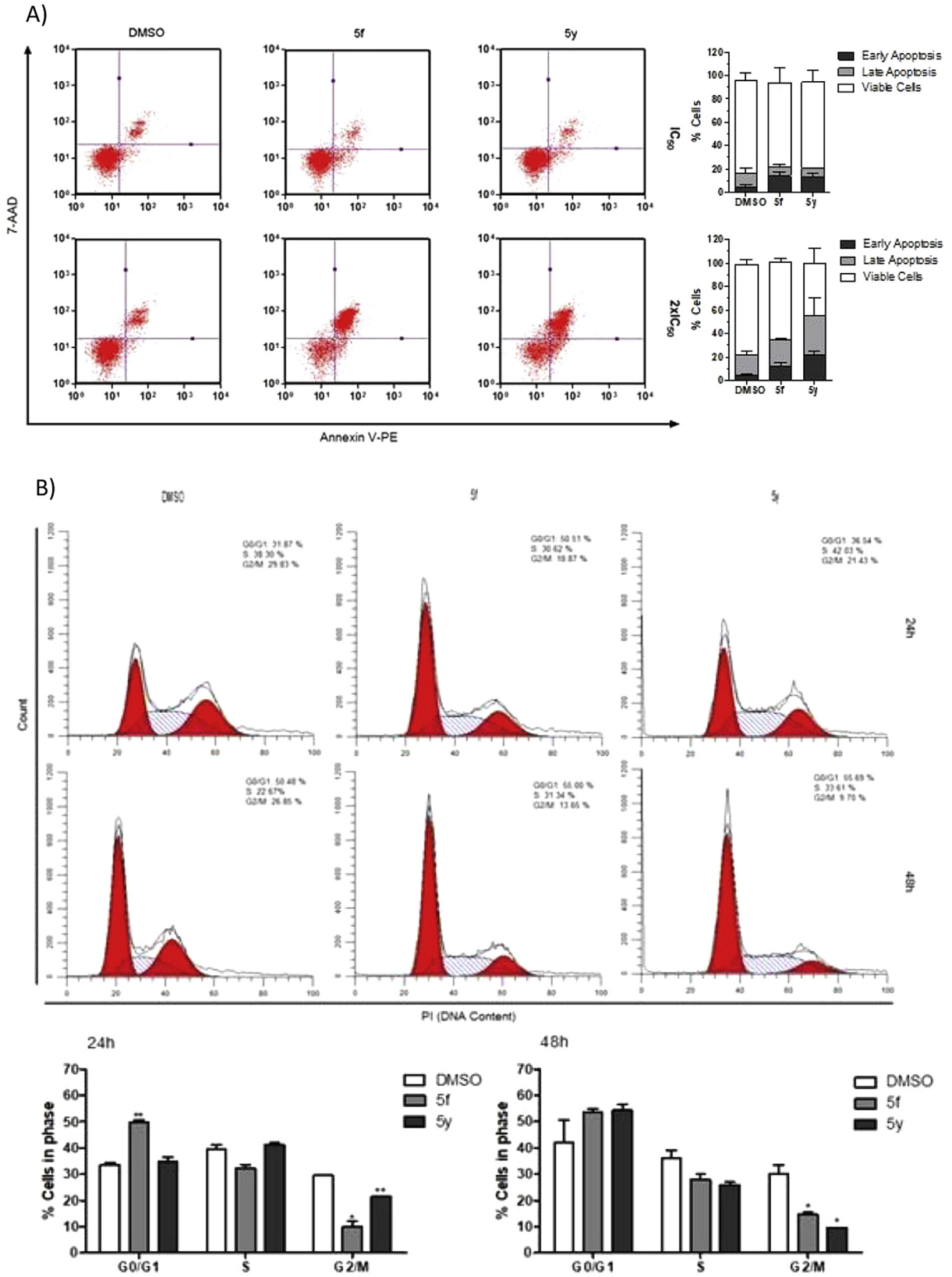


Fig. 4. Compounds **5f** and **5y** induce apoptosis and cell cycle arrest in the HCT-116 $p53^{(+/+)}$ cell line. **A**. Apoptosis was investigated by flow cytometry using 7-aminoactinomycin D (7-AAD) and Annexin-V-FITC. Cells were treated with either DMSO or tested compounds at IC_{50} or 2-fold IC_{50} and incubated for 72 h. Data represents percentage of apoptotic cells ± SD of three independent experiments. **B**. Cellular DNA was stained with propidium iodide (PI), and flow cytometry analysis was performed to determine cell cycle distribution. Cells were treated with either DMSO or tested compounds at IC_{50} and incubated for 24 and 48 h. Following flow cytometry analysis, frequencies of cells in each phase of the cell cycle were calculated using Mod Fit LT 4.1 software. Histograms show one representative example from at least two independent experiments (top). Results are expressed in the graph bar as means ± SD of at least two independent experiments (bottom).

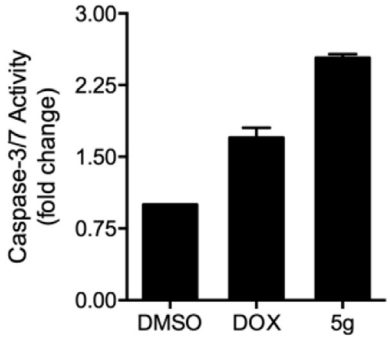


Fig. 5. - Compound **5g** induces caspase-3/-7 activity in MDA-MB-231 cells. Caspase-3 and -7 activities were measured using Caspase-Glo 3/7 luminescent assay (Promega). MDA-MB-231 cells were treated with DMSO, Doxorubicin or **5g** at IC_{50} and incubated for 48 h. Data represents mean ± SEM of two independent experiments.

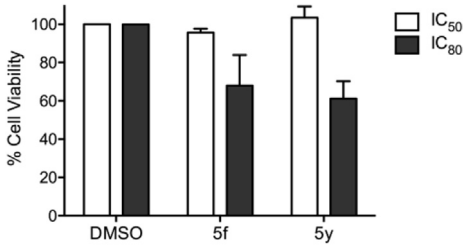


Fig. 6. Compounds **5f** and **5y** are non-cytotoxic to CCD-18Co cell line at their HCT-116 $p53^{(+/+)}$ IC_{50}. Cell viability in CCD-18Co fibroblasts was determined by the MTS method after 72 h for compounds **5f** and **5y** at their IC_{50} and IC_{80} in HCT116 $p53^{(+/+)}$. Data represent mean ± SEM of two independent experiments.

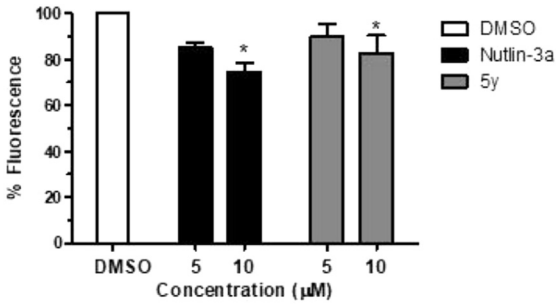


Fig. 7. Compound **5y** decreases p53–MDM2 interaction evaluated by the BiFC assay. HCT-116 $p53^{(-/-)}$ cells were co-transfected with V1-p53/MDM2-V2 BiFC combination plasmids for 24 h. Vehicle, nutlin-3a (5 and 10 μM) and compound **5y** (5 and 10 μM) were included in the culture medium 4 h after transfection. V1-p53/MDM2-V2 complementation was evaluated by flow cytometry. Results are expressed as the percentage (%) of fluorescence normalized to vehicle control DMSO (100% fluorescence intensity). Data represent mean ± SEM of three independent experiments. *$p < 0.05$ from control with DMSO.

applying a Venus-based bimolecular fluorescence complementation system methodology (BiFC) [34], we observed that compound **5y** was able to decrease the p53–MDM2 interaction at 10 μM (Fig. 7). Importantly, this effect may contribute for both apoptotic and cell cycle arrest outcomes observed for this compound.

Additionally, compound **5y** ability to disrupt p53-MDM2 binding was further corroborated by the significant decrease of MDM2 protein expression levels accompanied by an increase of p53 (Fig. 8).

3. Conclusion

Twenty-six compounds were synthesized with different substituents attached to the spirotriazoline oxindole scaffold. The antiproliferative activity of the synthesized compounds was assessed in four different cancer cell lines, and in the non-cancer derived HEK 293T cell line. Interestingly, the replacement of a pyrazoline ring by a triazoline ring in the spirooxindole core, changed the antiproliferative profile of spirotriazoline oxindoles **5** throughout the five cell lines tested. Specifically, we were able to achieve four spirotriazoline oxindoles (compounds **5g-j**) selective against MDA-MB-231 cells (≥2.4-fold) over the other four cell lines. In addition, four spirotriazoline oxindoles (compounds **5b**, **5f-g**, **5k**) showed selectivity towards all four cancer cell lines (≥2.5-fold) over the non-cancer derived cell line HEK 293T, with compound **5f** achieving a selectivity higher than 5.6-fold (highest cancer cell line IC_{50} = 17.9 μM vs. HEK 293T IC_{50} > 100 μM). The spiropyrazoline oxindole **4c**, with the same substitution pattern as the most potent

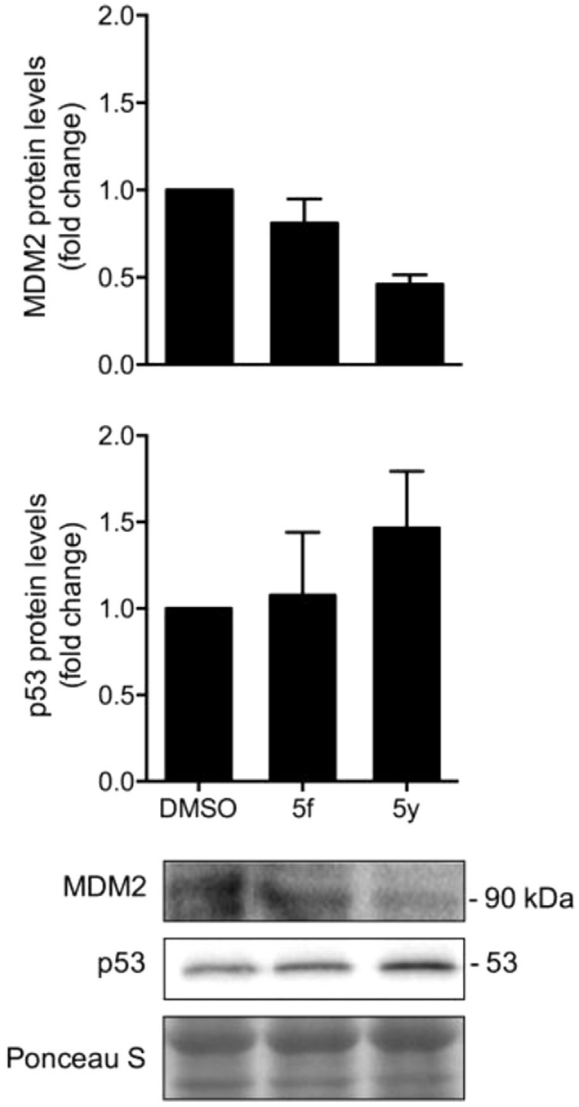


Fig. 8. Effect of compounds **5f** and **5y** in p53 and MDM2 protein levels. Total proteins were isolated from HCT-116 $p53^{(+/+)}$ cells following incubation with compounds **5f** and **5y** at equitoxic IC50 concentration, or DMSO (vehicle control), for 72 h. Ponceau S staining was used as loading control. Data represent mean ± SEM of two independent experiments.

compound in MDA-MB-231 cancer cell line (compounds **5i**), was found to be 3.6-fold less active in MDA-MD-231 cell line, and 2.2-fold more potent in MCF-7 cell line, while losing selectivity among all cancer cell lines, and HEK 293T cells.

The mechanism of action of spirotryazoline oxindoles as potential anti-cancer agents acting by a p53-dependent pathway was studied in more detail. Compounds **5f** and **5y** were not cytotoxic in normal cells, being selective for cancer cells over normal cells, while inducing apoptosis and cell cycle arrest in G0/G1 phase in the HCT-116 colorectal cancer cell line. Additionally, compounds **5f** and **5y** slightly induced p53 and decreased MDM2 expression levels. These changes in p53 and MDM2 expression levels, together with the results obtained in the BiFC assay strongly suggest the ability of spirotryazoline oxindoles to modulate p53-MDM2 interaction.

Furthermore, four spirotriazoline oxindoles derivatives (**5g-j**, $IC_{50} = 3.5–6.7$ μM) were selective against the TNBC cell line MDA-MB-231 (SI > 2.4-fold) over the other four cell lines, with compound **5g** exhibiting 8.4-fold selectivity against MDA-MB-231 over HEK 293T cells. Moreover, exposure of MDA-MB-231 cells to **5g** resulted in activation of effector caspases-3 and -7, thus suggesting that apoptosis is, at least in part, involved as the death mechanism. These four compounds have the common feature of containing a bromine at position 5 of the oxindole, and unsubstituted phenyl rings B and C. We are currently investigating in more detail the selectivity of these compounds against the more challenging TNBC cell line.

4. Experimental section

4.1. Chemistry: general conditions

All reagents and solvents were obtained from commercial suppliers and were used without further purification, with exception of NEt_3 and reaction solvents, which were dried prior to their use. 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. Merck Silica Gel 60 F_{254} 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). Preparative TLC was performed on Merck Silica Gel 60 GF_{254} over glass plates with 1 mm thickness. ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield at 400 MHz (^1H NMR) and 100 MHz (^{13}C NMR), and on a Bruker 300 Avance at 300 MHz (^1H NMR) and 75 MHz (^{13}C NMR). Data are reported as follows: chemical shift (δ), multiplicity (s: singlet, d: doublet, dd: doublet of doublet; t: triplet, m: multiplet, br: broad), coupling constants (J) in Hertz and integration. ^1H and ^{13}C chemical shifts are expressed in ppm using the solvent as internal reference. All compounds tested showed appropriate purity by elemental analysis (C, H, and N), performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK) at Liquid Chromatography and Mass Spectrometry Laboratory, Faculty of Pharmacy of Universidade de Lisboa.

4.1.1. Synthesis of hydrazones

A mixture of phenylhydrazine derivative (1.0 equiv), and benzaldehyde derivative (1.2 equiv) in aqueous ethanol 20% (2 mL/mmol of phenylhydrazine) was stirred at room temperature in the dark for 2–3 h. The precipitate formed was filtered and washed with aqueous ethanol 20%. For compounds **10f-g**, and **10i**, the starting phenylhydrazine was purchased in its chloridrate form, and therefore triethylamine (1.0 equiv) was added 15 min before adding the corresponding benzaldehyde. Spectroscopic characteristics of hydrazones **10a** [35], **10b** [36], **10c-d** [35], and **10g** [37], have been reported before (adapted from Ref. [30]).

*4.1.1.1. 1-Benzylidene-2-(2-chlorophenyl)hydrazine (**10e**).* Light pink solid (2.4 g, 92%). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.08 (br s, 1H, NH), 7.84 (s, 1H), 7.73–7.66 (m, 2H), 7.64 (dd, J = 8.2, 1.4 Hz, 1H), 7.44–7.31 (m, 3H), 7.31–7.21 (m, 2H), 6.87–6.75 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 140.68 (Cq), 139.64 (CH), 135.05 (Cq), 129.23(CH), 129.00 (CH), 128.80 (2CH), 128.09 (CH), 126.55 (2CH), 120.14 (CH), 117.01 (Cq), 114.33 (CH).

*4.1.1.2. 1-Benzylidene-2-(2-chlorophenyl)hydrazine (**10f**).* Off-white solid (2.5 g, 84% yield). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.70–7.62 (m, 3H), 7.59 (br s, 1H, NH), 7.45–7.28 (m, 3H), 7.23–7.11 (m, 2H), 6.91 (dd, J = 8.2, 1.2 Hz, 1H), 6.84 (dd, J = 7.9, 1.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 145.86 (Cq), 138.48 (CH), 135.28 (Cq), 134.98 (Cq), 130.39 (CH), 128.93 (CH), 128.79 (2CH), 126.48 (2CH), 120.03 (CH), 112.85 (CH), 111.00 (CH).

*4.1.1.3. 1-(3-chlorobenzylidene)-2-(2-chlorophenyl)hydrazine (**10h**).* White solid (2.7 g, 90% yield). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.15 (br s, 1H, NH), 7.76 (s, 1H), 7.71 (s, 1H), 7.63 (dd, J = 8.2, 1.4 Hz, 1H), 7.53–7.47 (m, 1H), 7.35–7.22 (m, 4H), 6.87–6.77 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 140.30 (Cq), 137.74 (CH), 136.91 (Cq), 134.83 (Cq), 129.99 (CH), 129.26 (CH), 128.77 (CH), 128.12 (CH), 126.01 (CH), 124.80 (CH), 120.51 (CH), 117.12 (Cq), 114.40 (CH).

*4.1.1.4. 1-(3-chlorobenzylidene)-2-(3-chlorophenyl)hydrazine (**10i**).* Light pink solid (2.6 g, 88% yield). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.74 (br s, 1H, NH), 7.66 (s, 1H), 7.63 (s, 1H), 7.53–7.47 (m, 1H), 7.32–7.23 (m, 2H), 7.22–7.14 (m, 2H), 6.93 (ddd, J = 8.3, 2.1, 0.9 Hz, 1H), 6.85 (ddd, J = 7.9, 2.0, 0.9 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 145.48 (Cq), 136.87 (Cq), 136.62 (CH), 135.34 (Cq), 134.85 (Cq), 130.45 (CH), 130.02 (CH), 128.74 (CH), 126.06 (CH), 124.66 (CH), 120.44 (CH), 112.97 (CH), 111.11 (CH).

4.1.2. General procedure for the synthesis of hydrazonoyl chlorides

To NCS (3.0 equiv) in CH_2Cl_2 (3.5 mL/mmol of hydrazone) at 0 °C was added methyl sulfide (6.0 equiv) over 5 min. After stirring for 15 min, the reaction was further cooled to −78 °C. Then the corresponding hydrazone **10** (1.0 equiv) dissolved in CH_2Cl_2 (1 mL/mmol of hydrazone) was added. The reaction was stirred at −78 °C for 1 h, and then slowly allowed to warm to room temperature over 3 h. The reaction was quenched by addition of cold water. The organic layer was then washed with brine (1x), saturated sodium sulfite aqueous solution (2x), and water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to afford the corresponding hydrazonoyl chloride (adapted from Ref. [32]). Spectroscopic characteristics of hydrazonoyl chlorides **7a** [38], **7b-d** [32], **7f** [32], and **7g** [39] have been reported before.

*4.1.2.1. N'-(2-chlorophenyl)benzohydrazonoyl chloride (**7e**).* Pink solid (1.9 g, 84% yield). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.57 (br s, 1H, NH), 7.98–7.91 (m, 2H), 7.60 (dd, J = 8.2, 1.5 Hz, 1H), 7.47–7.37 (m, 3H), 7.33 (dd, J = 8.0, 1.4 Hz, 1H), 7.30–7.22 (m, 1H), 6.87 (ddd, J = 7.9, 7.5, 1.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 139.52 (Cq), 134.31 (Cq), 129.71 (CH), 129.42 (CH), 128.59 (2CH), 128.12 (CH), 127.37 (Cq), 126.74 (2CH), 121.23 (CH), 118.14 (Cq), 114.75 (CH).

*4.1.2.2. 3-Chloro-N'-(2-chlorophenyl)benzohydrazonoyl chloride (**7h**).* Dark pink-reddish solid (1.6 g, 73% yield). ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.59 (br s, 1H, NH), 7.94–7.92 (m, 1H), 7.85–7.79 (m, 1H), 7.59 (dd, J = 8.2, 1.5 Hz, 1H), 7.37–7.24 (m, 4H), 6.93–6.85 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 139.17 (Cq), 136.00 (Cq), 134.71 (Cq), 129.79 (CH), 129.59 (CH), 129.47 (CH), 128.17 (CH),

126.55 (CH), 125.70 (Cq), 124.78 (CH), 121.61 (CH), 118.29 (Cq), 114.83 (CH).

*4.1.2.3. 3-Chloro-N'-(3-chlorophenyl)benzohydrazonoyl chloride (**7i**).* Dark brown-reddish solid (1.6 mg, 73% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.08 (br s, 1H, NH), 7.91–7.88 (m, 1H), 7.83–7.77 (m, 1H), 7.38–7.34 (m, 2H), 7.24–7.19 (m, 2H), 7.01 (ddd, J = 8.3, 2.2, 0.9 Hz, 1H), 6.93 (ddd, J = 8.0, 1.9, 0.9 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 144.20 (Cq), 135.92 (Cq), 135.44 (Cq), 134.72 (Cq), 130.56 (CH), 129.80 (CH), 129.57 (CH), 126.52 (CH), 124.71 (CH), 124.41 (Cq), 121.54 (CH), 113.74 (CH), 111.85 (CH).

4.1.3. Synthesis of 3-imino-indoline-2-ones

A mixture of indoline-2,3-dione derivative **8** (1.0 equiv), aniline derivative **9** (1.0 equiv), and acetic acid (0.1 equiv) in ethanol (4.0 mL/mmol of indoline-2,3-dione derivative) was heated at reflux for 3–5 h, under nitrogen atmosphere. If after 5 h the reaction was not completed an additional 0.5 equiv of aniline derivative **9** was added and refluxed to maximum reaction duration of 24 h. The reaction mixture was allowed to cool to room temperature, and the residue was filtrated. The solid obtained was washed with cold ethanol (adapted from Ref. [40]). Spectroscopic characteristics of 3-imino-indoline-2-ones **6a** [41,42], **6b** [43], **6c** [44], and **6d** [45] have been reported before.

*4.1.3.1. 5-Chloro-3-(phenylimino)indolin-2-one (**6e**).* Dark yellow solid (1.2 g, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 9.18 (br s, 1H, NH), 7.47 (t, J = 7.8 Hz, 2H), 7.33–7.27 (m, 2H), 7.02 (d, J = 7.8 Hz, 2H), 6.88 (d, J = 8.3 Hz, 1H), 6.64 (d, J = 1.4 Hz, 1H).

*4.1.3.2. 5-Chloro-3-((3-chlorophenyl)imino)indolin-2-one (**6f**).* Dark orange solid (1.1 g, 68% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.43 (br s, 1H, NH), 7.40 (t, J = 7.9 Hz, 1H), 7.33 (dd, J = 8.4, 1.6 Hz, 1H), 7.28 (d, J = 7.8 Hz, 1H), 7.04 (s, 1H), 6.91–6.86 (m, 2H), 6.67 (s, 1H).

*4.1.3.3. 5-Bromo-3-(phenylimino)indolin-2-one (**6g**).* Yellow solid (1.1 g, 80% yield).^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.44 (br s, 1H, NH), 7.50–7.42 (m, 3H), 7.30 (t, J = 7.6 Hz, 1H), 7.02 (d, J = 7.5 Hz, 2H), 6.81 (d, J = 8.4 Hz, 1H), 6.78 (s, 1H).

*4.1.3.4. 5-Bromo-3-((3-chlorophenyl)imino)indolin-2-one (**6h**).* Dark orange solid (698.3 mg, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.19 (br s, 1H, NH), 7.48 (d, J = 8.1 Hz, 1H), 7.40 (t, J = 7.9 Hz, 1H), 7.30–7.25 (m, 1H), 7.04 (s, 1H), 6.89 (d, J = 7.6 Hz, 1H), 6.86–6.79 (m, 2H).

*4.1.3.5. 5-Bromo-3-((4-chlorophenyl)imino)indolin-2-one (**6i**).* Orange solid (637.2 mg, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$ major (*E*) isomer δ (ppm): 8.07 (br s, 1H, NH), 7.50–7.40 (m, 3H), 7.05–6.96 (m, 2H), 6.93 (s, 1H), 6.86–6.78 (m, 1H).

*4.1.3.6. 5-bromo-3-((3-chloro-4-fluorophenyl)imino)indolin-2-one (**6j**).* Orange solid (641.3 mg, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.34 (br s, 1H, NH), 7.50 (d, J = 8.4 Hz, 1H), 7.29–7.23 (m, 1H), 7.16–7.10 (m, 1H), 6.96–6.88 (m, 2H), 6.84 (d, J = 8.2 Hz, 1H).

*4.1.3.7. 7-Chloro-3-(phenylimino)indolin-2-one (**6k**).*
Synthesized according to the general procedure. However, since compound **6k** did not precipitate after cooling to room temperature, the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using as eluent a gradient from 100% DCM to DCM:EtOAc (90:10). Yellow solid (308.0 mg, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.25 (br s, 1H, NH), 7.44 (t, J = 7.8 Hz, 2H, H_{Ar}), 7.32 (d, J = 8.1 Hz, 1H), 7.30–7.24 (m, 1H), 7.00 (d, J = 7.8 Hz, 2H), 6.72 (t, J = 8.0 Hz, 1H), 6.57 (d, J = 7.8 Hz, 1H).

*4.1.3.8. 7-Bromo-3-(phenylimino)indolin-2-one (**6l**).*
Synthesized according to **6k**. Yellow solid (245.0 mg, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 8.04 (br s, 1H, NH), 7.48–7.41 (m, 3H), 7.30–7.24 (m, 1H), 7.00 (d, J = 8.0 Hz, 2H), 6.67 (t, J = 7.7 Hz, 1H), 6.61 (d, J = 7.6 Hz, 1H).

*4.1.4. Synthesis of 5-bromo-3-(3-chlorobenzylidene)indolin-2-one (**13**)*

A mixture of 5-bromoindolin-2,3-dione (1.0 equiv), 3-chlorobenzaldehyde (1.2 equiv) and piperidine (0.1 equiv) in ethanol was refluxed for 8 h, under nitrogen atmosphere. After the reaction was complete, the solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel using as eluent a gradient from *n*-hexane/EtOAc (4:1) to *n*-hexane/EtOAc (2:1) to afford the final product, as a mixture of *E*/*Z* isomers (adapted from Ref. [46])

*4.1.4.1. Compound **13**.* Yellow solid (369.2 mg, 94% yield). ^{1}H NMR (300 MHz, $CDCl_3$) major (*E*) isomer δ (ppm): 7.84 (br s, 1H), 7.79 (s, 1H), 7.69 (d, J = 1.8 Hz, 1H), 7.61 (d, J = 0.9 Hz, 1H), 7.56–7.51 (m, 1H), 7.48–7.44 (m, 2H), 7.38 (dd, J = 8.3, 1.8 Hz, 1H), 6.79 (d, J = 8.3 Hz, 1H).

4.1.5. General procedure for the synthesis of 2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-ones

Triethylamine (2.0 equiv) was added dropwise to a mixture of 3-imino indolin-2-one derivative **6** (1.0 equiv), and hydrazonoyl chloride derivative **7** (2.0 equiv) in dry DCM (1 mL/0.1 mmol of 3-iminoidolin-2-one) under nitrogen atmosphere. The reaction was stirred at room temperature for 24 h. The mixture was then washed with brine (2x) and the aqueous phase extracted with DCM. 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 a gradient of *n*-hexane/EtOAc (95:5) to *n*-hexane/EtOAc (75:25), preparative thin layer chromatography (PTLC) on silica gel using as eluent *n*-hexane:EtOAc (2:1), and recrystallized from diethyl ether/*n*-hexane to afford the final product.

*4.1.5.1. 2′,4′,5′-triphenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5a**).* Synthesized according to the general procedure, and starting with **6a** and **7a**, this compound was obtained as a yellow solid (151.2 mg, 81% yield). Mp: 203–204 °C (Lit. 202 °C [28]); IR (KBr, selected peaks): 3245 (NH), 1735 (C=O), 1594 (C=N), 1493, 1472, 1385, 1320, 1203, 751, 694 cm^{-1}; ^{1}H NMR (400 MHz, Acetone-d_6) δ (ppm): 7.55 (d, J = 7.4 Hz, 1H), 7.48 (d, J = 7.0 Hz, 2H), 7.38–7.27 (m, 4H), 7.18–7.03 (m, 6H), 6.97–6.89 (m, 3H), 6.87–6.79 (m, 2H), 6.73 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.49 (Cq), 148.51 (Cq), 144.43 (Cq), 143.06 (Cq), 139.18, (Cq) 132.31 (CH), 130.07 (CH), 129.67 (2CH), 129.61 (2CH), 129.03 (2CH), 128.88 (Cq), 128.62 (2CH), 128.61 (2CH), 127.72 (CH), 127.34 (Cq), 127.26 (CH), 123.99 (CH), 120.33 (CH), 114.54 (2CH), 111.73 (CH), 88.46 (Cspiro). Anal. Calcd for $C_{27}H_{20}N_4O \cdot 0.25H_2O$: C 77.03, H 4.92, N 13.31, found: C 76.63, H 4.99, N 13.05.

*4.1.5.2. 4'-(4-chlorophenyl)-2′,5′-diphenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4] triazol]-2-one (**5b**).* Synthesized according to the general procedure, and starting with **6c** and **7a**, this compound was obtained as a yellow solid (146.9 mg, 84% yield). Mp: 185–187 °C. IR (KBr, selected peaks): 3243 (NH), 1738 (C=O), 1597 (C=N), 1491,

1471, 1385, 1320, 1200, 745, 691 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.68 (br s, 1H, NH), 7.56 (d, J = 7.4 Hz, 1H), 7.49 (d, J = 8.0 Hz, 2H), 7.40–7.30 (m, 4H), 7.18 (d, J = 8.6 Hz, 2H), 7.13–7.07 (m, 3H), 6.97–6.90 (m, 3H), 6.82 (d, J = 8.6 Hz, 2H), 6.74 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.21 (Cq), 148.15 (Cq), 144.30 (Cq), 142.98 (Cq), 138.14 (Cq), 132.73 (Cq), 132.51 (CH), 130.26 (CH), 130.04 (2CH), 129.76 (2CH), 129.65 (2CH), 129.18 (2CH), 128.61 (2CH), 128.52 (Cq), 127.26 (CH), 127.06 (Cq), 124.16 (CH), 120.55 (CH), 114.62 (2CH), 111.88 (CH), 88.42 (Cspiro). Anal. Calcd for $C_{27}H_{19}ClN_4O \cdot 0.65H_2O$: C 70.09, H 4.43, N 12.11, found: C 69.67, H 4.27, N 11.69.

*4.1.5.3. 4'-(3-chlorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4] triazol]-2-one (**5c**).* Synthesized according to the general procedure, and starting with **6b** and **7a**, this compound was obtained as a yellow solid (158.2 mg, 90% yield). Mp: 208–209 C; IR (KBr, selected peaks): 3255 (NH), 1735 (C=O), 1591 (C=N), 1494, 1470, 1384, 1320, 1198, 753, 689 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.72 (br s, 1H, NH), 7.57 (d, J = 7.3 Hz, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.43–7.32 (m, 4H), 7.20–7.14 (m, 2H), 7.13–7.08 (m, 3H), 6.98 (d, J = 7.8 Hz, 1H), 6.94 (d, J = 7.9 Hz, 2H), 6.81 (s, 1H), 6.80–6.71 (m, 2H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.11 (Cq), 147.91 (Cq), 144.25 (Cq), 142.92 (Cq), 140.71 (Cq), 134.49 (Cq), 132.59 (CH), 131.02 (CH), 130.34 (CH), 129.67 (2CH), 129.23 (2CH), 128.62 (2CH), 128.51 (Cq), 127.97 (CH), 127.61 (CH), 127.28 (CH), 126.90 (Cq), 126.86 (CH), 124.20 (CH), 120.67 (CH), 114.73 (2CH), 111.87 (CH), 88.36 (Cspiro). Anal. Calcd for $C_{27}H_{19}ClN_4O \cdot 0.6H_2O$: C 70.23, H 4.42, N 12.14, found: C 69.96, H 4.32, N 11.92.

*4.1.5.4. 4'-(3-chloro-4-fluorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5d**).* Synthesized according to the general procedure, and starting with **6d** and **7a**, this compound was obtained as a yellow solid (119.5 mg, 70% yield). Mp: 203–205 °C; IR (KBr, selected peaks): 3246 (NH), 1731 (C=O), 1597 (C=N), 1493, 1470, 1385, 1325, 1203, 746, 690 cm^{-1}; ^1H NMR (400 MHz, Acetone.d_6) δ (ppm): 9.72 (br s, 1H, NH), 7.61 (d, J = 7.3 Hz, 1H), 7.52 (d, J = 8.0 Hz, 2H), 7.44–7.32 (m, 4H), 7.17–7.07 (m, 4H), 6.98 (d, J = 7.8 Hz, 1H), 6.96–6.90 (d, J = 8.0 Hz, 3H), 6.87–6.80 (m, 1H), 6.76 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.13 (Cq), 157.26 (d, J_{FC} = 247.0 Hz, Cq), 147.92 (Cq), 144.26 (Cq), 142.98 (Cq), 136.29 (d, J_{FCCCC} = 3.0 Hz, Cq), 132.67 (CH), 130.59 (CH), 130.41 (CH), 129.69 (2CH), 129.37 (d, J_{FCCC} = 8.0 Hz, CH) 129.28 (2CH), 128.67 (2CH), 128.27 (Cq), 127.33 (CH), 126.83 (Cq), 124.27 (CH), 121.02 (d, J_{FCC} = 19.0 Hz, Cq), 120.71 (CH), 117.66 (d, J_{FCC} = 22.0 Hz, CH), 114.68 (2CH), 111.96 (CH), 88.52 (Cspiro). Anal. Calcd for $C_{27}H_{18}ClFN_4O \cdot 0.3H_2O$: C 68.37, H 3.96, N 11.81, found: C 68.75, H 3.62, N 11.42.

*4.1.5.5. 5-Chloro-2',4',5'-triphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5e**).* Synthesized according to the general procedure, and starting with **6e** and **7a**, this compound was obtained as a yellow solid (153.3 mg, 87% yield). Mp: 218–220 °C; IR (KBr, selected peaks): 3244 (NH), 1742 (C=O), 1595 (C=N), 1493, 1476, 1384, 1320, 1201, 764, 693 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.74 (br s, 1H, NH), 7.64 (s, 1H), 7.50 (d, J = 7.7 Hz, 2H), 7.42–7.29 (m, 4H), 7.21–7.11 (m, 5H), 6.99–6.93 (m, 3H), 6.91 (d, J = 7.2 Hz, 2H), 6.78 (t, J = 7.2 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.33 (Cq), 148.59 (Cq), 144.20 (Cq), 141.76 (Cq), 138.93 (Cq), 132.27 (CH), 130.18 (CH), 129.82 (2CH), 129.74 (2CH), 129.20 (Cq), 129.04 (2CH), 128.82 (Cq), 128.69 (4CH), 128.63 (Cq), 127.99 (CH), 127.33 (CH), 120.65 (CH), 114.58 (2CH), 113.22 (CH), 88.38 (Cspiro). Anal. Calcd for $C_{27}H_{19}ClN_4O \cdot 0.75H_2O$: C 69.69, H 4.47, N 12.04, found: C 69.82, H 4.46, N 12.07.

*4.1.5.6. 5-Chloro-4'-(3-chlorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5f**).* Synthesized according to the general procedure, and starting with **6f** and **7a**, this compound was obtained as a yellow solid (148.4 mg, 89% yield). Mp: 172–173 °C. IR (KBr, selected peaks): 3252 (NH), 1741 (C=O), 1590 (C=N), 1495, 1477, 1385, 1323, 1199, 745, 689 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.86 (br s, 1H, NH), 7.65 (d, J = 1.7 Hz, 1H), 7.56–7.48 (m, 2H), 7.44–7.32 (m, 4H), 7.25–7.16 (m, 2H), 7.13 (t, J = 8.0 Hz, 2H), 7.01 (d, J = 8.2 Hz, 1H), 6.94 (d, J = 8.0 Hz, 2H), 6.89 (s, 1H), 6.86–6.81 (m, 1H), 6.78 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.05 (Cq), 148.01 (Cq), 144.04 (Cq), 141.73 (Cq), 140.48 (Cq), 134.62 (Cq), 132.54 (CH), 131.18 (CH), 130.43 (CH), 129.80 (2CH), 129.22 (2CH), 129.03 (Cq), 128.79 (Cq), 128.72 (2CH), 128.30 (Cq), 128.14 (CH), 127.91 (CH), 127.39 (CH), 126.96 (CH), 120.97 (CH), 114.77 (2CH), 113.40 (CH), 88.28 (Cspiro). Anal. Calcd for $C_{27}H_{18}Cl_2N_4O \cdot 0.5H_2O$: C 65.59, H 3.88, N 11.34, found: C 65.19, H 4.23, N 11.31.

*4.1.5.7. 5-Bromo-2',4',5'-triphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5g**).* Synthesized according to the general procedure, and starting with **6g** and **7a**, this compound was obtained as a yellow solid (155.7 mg, 95% yield). Mp: 212–214 °C. IR (KBr, selected peaks): 3211 (NH), 1741 (C=O), 1595 (C=N), 1493, 1473, 1385, 1320, 1200, 766, 693 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.74 (br s, 1H, NH), 7.75 (s, 1H), 7.52 (d, J = 8.6 Hz, 1H), 7.48 (d, J = 7.6 Hz, 2H), 7.38–7.27 (m, 3H), 7.20–7.10 (m, 5H), 6.96–6.85 (m, 5H), 6.76 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.13 (Cq), 148.59 (Cq), 144.18 (Cq), 142.19 (Cq), 138.92 (Cq), 135.18 (CH), 130.18 (CH), 130.12 (CH), 129.83 (2CH), 129.76 (2CH), 129.56 (Cq), 129.04 (2CH), 128.70 (4CH), 128.62 (Cq), 128.01 (CH), 120.65 (CH), 116.04 (Cq), 114.56 (2CH), 113.68 (CH), 88.32 (Cspiro). Anal. Calcd for $C_{27}H_{19}BrN_4O$: C 65.46, H 3.87, N 11.31, found: C 65.31, H 3.97, N 11.05.

*4.1.5.8. 5-Bromo-4'-(4-chlorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5h**).* Synthesized according to the general procedure, and starting with **6i** and **7a**, this compound was obtained as a yellow solid (146.1 mg, 93% yield). Mp: 140–142 °C; IR (KBr, selected peaks): 3186, 1730, 1598, 1490, 1473, 1385, 1307, 1205, 747, 690 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.09 (br s, 1H, NH), 7.70 (s, 1H), 7.50–7.41 (m, 3H), 7.37–7.22 (m, 3H), 7.12 (t, J = 7.7 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 6.90 (d, J = 8.0 Hz, 2H), 6.83 (t, J = 7.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 2H), 6.64 (d, J = 8.3 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 173.21 (Cq), 147.96 (Cq), 143.10 (Cq), 139.72 (Cq), 136.41 (Cq), 134.63 (CH), 133.04 (Cq), 129.78 (CH), 129.58 (CH), 129.42 (2CH), 129.29 (2CH), 129.05 (Cq), 128.83 (2CH), 128.49 (2CH), 128.03 (2CH), 126.91 (Cq), 120.99 (CH), 116.85 (Cq), 114.49 (2CH), 113.07 (CH), 88.09 (Cspiro). Anal. Calcd for $C_{27}H_{18}BrClN_4O \cdot 0.05H_2O$: C 61.10, H 3.44, N 10.56, found: C 60.71, H 3.50, N 10.23.

*4.1.5.9. 5-Bromo-4'-(3-chlorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5i**).* Synthesized according to the general procedure, and starting with **6h** and **7a**, this compound was obtained as a yellow solid (144.8 mg, 92% yield). Mp: 143–145 °C; IR (KBr, selected peaks): 328 (NH), 1738 (C=O), 1597 (C=N), 1493, 1385, 1325, 1201, 745, 687 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.88 (br s, 1H, NH), 7.78 (d, J = 1.7 Hz, 1H), 7.56 (dd, J = 8.4, 1.7 Hz, 1H), 7.52 (d, J = 8.2 Hz, 2H), 7.42–7.33 (m, 3H), 7.24–7.17 (m, 2H), 7.14 (t, J = 7.8 Hz, 2H), 6.97 (d, J = 8.4 Hz, 1H), 6.93 (d, J = 7.8 Hz, 2H), 6.89 (s, 1H), 6.86–6.81 (m, 1H), 6.78 (t, J = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.90 (Cq), 148.04 (Cq), 144.04 (Cq), 142.19 (Cq), 140.49 (Cq), 135.47 (CH), 134.64 (Cq), 131.20 (CH), 130.45 (CH), 130.21 (CH), 129.82 (2CH), 129.24 (2CH), 129.15 (Cq), 128.74 (2CH), 128.31 (Cq), 128.18

(Cq), 127.94 (CH), 126.98 (CH), 120.98 (CH), 116.24 (Cq), 114.77 (2CH), 113.85 (CH), 88.23 (Cspiro). Anal. Calcd for $C_{27}H_{18}BrClN_4O \cdot 0.15H_2O$: C 60.89, H 3.47, N 10.52, Found: C 60.76, H 3.87, N 10.19.

*4.1.5.10. 5-Bromo-4'-(3-chloro-4-fluorophenyl)-2′,5′-diphenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5j**).* Synthesized according to the general procedure, and starting with **6j** and **7a**, this compound was obtained as a yellow solid (138.9 mg, 90% yield). MP: 135–137 °C; IR (KBr, selected peaks): 3242 (NH), 1735 (C=O), 1597 (C=N), 1492, 1473, 1384, 1261, 1194, 764, 691 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.75 (br s, 1H, NH), 7.82 (s, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.52 (d, J = 7.2 Hz, 2H), 7.42–7.32 (m, 3H), 7.20–7.10 (m, 3H), 7.03 (dd, J = 6.5, 2.2 Hz, 1H), 6.96 (d, J = 8.4 Hz, 1H), 6.95–6.88 (m, 3H), 6.78 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 172.84 (Cq), 157.42 (d, J_{FC} = 247.0 Hz, Cq), 148.03 (Cq), 144.02 (Cq), 142.14 (Cq), 136.02 (d, J_{FCCCC} = 4.0 Hz, Cq), 135.51 (CH), 130.83 (CH), 130.49 (CH), 130.22 (CH), 129.82 (2CH), 129.48 (d, J_{FCCC} = 7.0 Hz, CH), 129.27 (2CH), 129.04 (Cq), 128.78 (2CH), 128.04 (Cq), 121.17 (d, J_{FCC} = 18.0 Hz, Cq), 120.99 (CH), 117.82 (d, J_{FCC} = 23.0 Hz, CH), 116.29 (Cq), 114.70 (2CH), 113.91 (CH), 88.35 (Cspiro). Anal. Calcd for $C_{27}H_{18}BrClFN_4O \cdot 0.1H_2O$: C 59.00, H 3.16, N 10.20, Found: C 58.67, H 3.52, N 10.17.

*4.1.5.11. 7-Chloro-2′,4′,5′-triphenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5k**).* Synthesized according to the general procedure, and starting with **6k** and **7a**, this compound was obtained as a yellow solid (159.6 mg, 91% yield). Mp: 186–188 °C; IR (KBr, selected peaks): 3059 (NH), 1750 (C=O), 1594 (C=N), 1494, 1475, 1337, 1177, 1154, 749, 694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.53–7.44 (m, 3H), 7.32 (d, J = 8.2 Hz, 1H), 7.29 (d, J = 7.2 Hz, 1H), 7.27–7.21 (m, 2H), 7.19–7.02 (m, 6H), 6.91 (d, J = 8.0 Hz, 2H), 6.82 (t, J = 7.3 Hz, 1H), 6.80–6.72 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 172.37 (Cq), 148.38 (Cq), 143.34 (Cq), 138.47 (Cq), 137.86 (Cq), 131.27 (CH), 129.48 (CH), 129.20 (2CH), 129.17 (2CH), 128.87 (Cq), 128.27 (2CH), 128.07 (2CH), 127.77 (2CH), 127.38 (Cq), 127.32 (CH), 125.02 (CH), 124.74 (CH), 120.86 (CH), 116.08 (Cq), 114.80 (2CH), 88.97 (Cspiro). Anal. Calcd for $C_{27}H_{19}ClN_4O \cdot 0.65H_2O$: C 69.69, H 4.47, N 12.04, found: C 69.37, H 4.31, N 11.73.

*4.1.5.12. 7-Bromo-2′,4′,5′-triphenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5l**).* Synthesized according to the general procedure, and starting with **6l** and **7a**, this compound was obtained as a yellow solid (154.1 mg, 94% yield). Mp: 220–222 °C; IR (KBr, selected peaks): 3057 (NH), 1741 (C=O), 1594 (C=N), 1494, 1472, 1385, 1337, 1178, 761, 694 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.86 (br s, 1H, NH), 7.58 (d, J = 7.4 Hz, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 7.1 Hz, 2H), 7.38–7.28 (m, 3H), 7.20–7.09 (m, 5H), 7.06 (t, J = 7.7 Hz, 1H), 6.92 (d, J = 8.0 Hz, 2H), 6.87–6.81 (m, 2H), 6.76 (t, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 173.40 (Cq), 148.62 (Cq), 144.22 (Cq), 142.26 (Cq), 138.87 (Cq), 135.10 (CH), 130.21 (CH), 129.84 (2CH), 129.76 (2CH), 129.25 (Cq), 129.07 (2CH), 128.70 (2CH), 128.64 (2CH), 128.59 (Cq), 128.07 (CH), 126.36 (CH), 125.56 (CH), 120.73 (CH), 114.70 (2CH), 104.43 (Cq), 89.29 (Cspiro). Anal. Calcd for $C_{27}H_{19}BrN_4O$: C 65.46, H 3.87, N 11.31, found: C 65.36, H 3.99, N 11.18.

*4.1.5.13. 5'-(3-chlorophenyl)-4'-(4-chlorophenyl)-2′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5m**).* Synthesized according to the general procedure, and starting with **6c** and **7b**, this compound was obtained as a yellow solid (169.0 mg, 89% yield). Mp: 98–100 °C. IR (KBr, selected peaks): 3244 (NH), 1733 (C=O), 1593 (C=N), 1491, 1471, 1318, 1197, 1093, 746, 688 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.72 (br s, 1H, NH), 7.61–7.54 (m, 2H), 7.44–7.31 (m, 4H), 7.24–7.16 (m, 2H), 7.15–7.05 (m, 3H), 6.98–6.91 (m, 3H), 6.89–6.82 (m, 2H), 6.81–6.73 (m, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.08 (Cq), 146.87 (Cq), 144.01 (Cq), 142.93 (Cq), 137.75 (Cq), 134.59 (Cq), 133.01 (Cq), 132.61 (CH), 130.92 (CH), 130.49 (Cq), 130.11 (CH), 130.02 (2CH), 129.91 (2CH), 129.70 (2CH), 128.23 (CH), 127.33 (CH), 126.89 (CH), 126.75 (Cq), 124.20 (CH), 120.87 (CH), 114.72 (2CH), 111.93, 88.60 (Cspiro). Anal. Calcd for $C_{21}H_{14}ClN_3O_2 \cdot 0.9H_2O$: C 64.65, H 3.99, N 11.17, found: C 64.43, H 3.83, N 11.47.

*4.1.5.14. 4′,5′-bis(4-chlorophenyl)-2′-phenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4] triazol]-2-one (**5n**).* Synthesized according to the general procedure, and starting with **6c** and **7c**, this compound was obtained as a yellow solid (163.5 mg, 86% yield). Mp: 155–157 °C; IR (KBr, selected peaks): 3205 (NH), 1731 (C=O), 1600 (C=N), 1492, 1474, 1319, 1203, 1014, 835, 746 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.68 (br s, 1H, NH), 7.56 (d, J = 7.4 Hz, 1H), 7.53–7.46 (m, 2H), 7.43–7.34 (m, 3H), 7.24–7.17 (m, 2H), 7.15–7.05 (m, 3H), 7.00–6.89 (m, 3H), 6.88–6.80 (m, 2H), 6.79–6.71 (m, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.13 (Cq), 147.20 (Cq), 144.15 (Cq), 143.02 (Cq), 137.89 (Cq), 135.58 (Cq), 132.96 (Cq), 132.61 (CH), 130.17 (2CH), 130.04 (2CH), 129.91 (2CH), 129.70 (2CH), 129.41 (2CH), 127.32 (CH and Cq), 126.90 (Cq), 124.20 (CH), 120.78 (CH), 114.70 (2CH), 111.94 (CH), 88.56 (Cspiro). Anal. Calcd for $C_{21}H_{14}ClN_3O_2 \cdot 0.75H_2O$: C 65.00, H 3.95, N 11.23, found: C 64.64, H 3.90, N 11.50.

*4.1.5.15. 2'-(2-chlorophenyl)-4'-(4-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5o**).* Synthesized according to the general procedure, and starting with **6c** and **7e**, this compound was obtained as a white solid (146.7 mg, 76% yield). Mp: 212–214 °C; IR (KBr, selected peaks): 3261 (NH), 1739 (C=O), 1616 (C=N), 1492, 1472, 1384, 1202, 756, 697 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.31 (br s, 1H, NH), 7.70 (dd, J = 8.1, 1.5 Hz, 1H), 7.58–7.50 (m, 2H), 7.45–7.32 (m, 3H), 7.29 (ddd, J = 8.1, 7.3, 1.5 Hz, 1H) 7.21–7.08 (m, 4H), 7.04 (ddd, J = 8.0, 7.3, 1.5 Hz, 1H), 6.95 (d, J = 7.0 Hz, 1H), 6.84–6.69 (m, 4H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.99 (Cq), 152.73 (Cq), 144.20 (Cq), 144.15 (Cq), 138.61 (Cq), 132.44 (Cq), 132.03 (CH), 130.59 (CH), 130.28 (CH), 129.93 (2CH), 129.65 (2CH), 129.65 (Cq), 129.21 (2CH), 129.00 (2CH), 128.72 (Cq), 128.48 (CH), 127.82 (2CH), 126.76 (CH), 124.92 (Cq), 122.59 (CH), 111.27 (CH), 89.65 (Cspiro). Anal. Calcd for $C_{27}H_{18}Cl_2N_4O \cdot 0.3H_2O$: C 66.07, H 3.83, N 11.42, found: C 65.77, H 3.81, N 11.17.

*4.1.5.16. 2'-(3-chlorophenyl)-4'-(4-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5p**).* Synthesized according to the general procedure, and starting with **6c** and **7f**, this compound was obtained as a yellow solid (172.0 mg, 91% yield). Mp: 120–122 °C; IR (KBr, selected peaks): 3255 (NH), 1735 (C=O), 1591 (C=N), 1491, 1474, 1385, 1324, 1196, 1092, 754 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.79 (br s, 1, NH), 7.59 (dd, J = 7.6, 0.7 Hz, 1H), 7.55–7.47 (m, 2H), 7.45–7.31 (m, 4H), 7.23–7.17 (m, 2H), 7.12 (td, J = 7.6, 1.0 Hz, 1H), 7.11–7.04 (m, 2H), 6.98 (d, J = 7.7 Hz, 1H), 6.87–6.80 (m, 2H), 6.75 (ddd, J = 8.0, 2.0, 0.9 Hz, 1H), 6.67 (ddd, J = 8.4, 2.3, 0.9 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.86 (Cq), 149.09 (Cq), 145.34 (Cq), 143.01 (Cq), 137.78 (Cq), 135.19 (Cq), 133.08 (Cq), 132.87 (CH), 131.14 (CH), 130.61 (CH), 130.22 (2CH), 129.90 (2CH), 129.28 (2CH), 128.83 (2CH), 128.16 (Cq), 127.36 (CH), 126.33 (Cq), 124.40 (CH), 119.99 (CH), 114.39 (CH), 112.27 (CH), 112.06 (CH), 88.12 (Cspiro). Anal. Calcd for $C_{27}H_{18}Cl_2N_4O \cdot 0.4H_2O$: C 65.83, H 3.85, N 11.38, found: C 65.51, H 3.76, N 11.12.

*4.1.5.17. 2′,4′-bis(4-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4] triazol]-2-one (**5q**).* Synthesized according to the general procedure, and starting with **6c** and **7g**, this compound was obtained as a yellow solid (159.3 mg, 84% yield). MP: 130–132 °C; IR (KBr, selected peaks): 3208 (NH), 1731 (C=O), 1595 (C=N), 1490, 1385, 1320, 1194, 1014, 820, 746 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.68 (br s, 1H, NH), 7.57 (d, J = 7.4 Hz, 1H), 7.53–7.46 (m, 2H), 7.42–7.30 (m, 4H), 7.22–7.16 (m, 2H), 7.16–7.07 (m, 3H), 6.96 (d, J = 7.8 Hz, 1H), 6.94–6.86 (m, 2H), 6.86–6.78 (m, 2H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.93 (Cq), 148.85 (Cq), 143.19 (Cq), 143.03 (Cq), 137.95 (Cq), 132.98 (Cq), 132.75 (CH), 130.50 (CH), 130.15 (2CH), 129.86 (2CH), 129.56 (2CH), 129.25 (2CH), 128.76 (2CH), 128.32 (Cq), 127.35 (CH), 126.56 (Cq), 124.87 (Cq), 124.32 (CH), 116.05 (2CH), 112.07 (CH), 88.41 (Cspiro). Anal. Calcd for $C_{21}H_{14}ClN_3O_2$: C 66.81, H 3.75, N 11.55, found: C 66.60, H 3.76, N 11.36.

*4.1.5.18. 2'-(2-chlorophenyl)-5'-(3-chlorophenyl)-4'-(4-chlorophenyl)-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5r**).* Synthesized according to the general procedure, and starting with **6c** and **7h**, this compound was obtained as a white solid (173.8 mg, 86% yield). Mp: 244–245 °C. IR (KBr, selected peaks): 3284 (NH), 1745 (C=O), 1605 (C=N), 1492, 1473, 1427, 1375, 1195, 753, 688 cm^{-1}. ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.37 (br s, 1H, NH), 7.70 (dd, J = 8.1, 1.5 Hz, 1H), 7.65–7.60 (m, 1H), 7.46–7.33 (m, 3H), 7.29 (ddd, J = 8.1, 7.2, 1.6 Hz, 1H), 7.20–7.11 (m, 4H), 7.05 (ddd, J = 8.0, 7.2, 1.6 Hz, 1H), 6.97 (dd, J = 7.5, 1.5 Hz, 1H), 6.88–6.81 (m, 2H), 6.80–6.70 (m, 2H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.93 (Cq), 151.35 (Cq), 144.14 (Cq), 143.77 (Cq), 138.27 (Cq), 134.65 (Cq), 132.71 (Cq), 132.15 (CH), 130.98 (CH), 130.74 (Cq), 130.48 (CH), 130.34 (CH), 129.91 (2CH), 129.82 (2CH), 129.70 (Cq), 128.69 (CH), 128.57 (CH), 127.97 (CH), 127.88 (CH), 127.35 (CH), 127.01 (CH), 124.55 (Cq), 122.63 (CH), 111.30 (CH), 89.83 (Cspiro). Anal. Calcd for $C_{27}H_{17}Cl_3N_4O$: C 62.38, H 3.30, N 10.78, Found: C 62.78, H 3.41, N 10.65.

*4.1.5.19. 5-Bromo-4′,5′-bis(3-chlorophenyl)-2′-phenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5s**).* Synthesized according to the general procedure, and starting with **6j** and **7b**, this compound was obtained as a yellow solid (142.5 mg, 85% yield). Mp: 104–106 °C; IR (KBr, selected peaks): 3251 (NH), 1738 (C=O), 1590 (C=N), 1474, 1434, 1384, 1314, 1197, 748, 689 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.91 (br s, 1H, NH), 7.81 (d, J = 2.0 Hz, 1H), 7.61 (d, J = 0.9 Hz, 1H), 7.55 (dd, J = 8.3, 2.0 Hz, 1H), 7.45–7.34 (m, 3H), 7.26–7.20 (m, 2H), 7.15 (t, J = 8.0 Hz, 2H), 7.00–6.91 (m, 4H), 6.90–6.85 (m, 1H), 6.80 (t, J = 7.3 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.79 (Cq), 146.77 (Cq), 143.77 (Cq), 142.16 (Cq), 140.13 (Cq), 135.56 (CH), 134.79 (Cq), 134.64 (Cq), 131.36 (CH), 130.97 (CH), 130.32 (2CH), 130.30 (Cq), 129.87 (2CH), 128.82 (Cq), 128.42 (CH), 128.22 (CH), 128.19 (CH), 127.06 (CH), 127.04 (CH), 121.27 (CH), 116.29 (Cq), 114.87 (2CH), 113.88 (CH), 88.42 (Cspiro). Anal. Calcd for $C_{27}H_{17}BrCl_2N_4O$: C 57.47, H 3.04, N 9.93, Found: C 57.80, H 3.23, N 9.72.

*4.1.5.20. 5-Bromo-4'-(3-chlorophenyl)-5'-(4-chlorophenyl)-2′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5t**).* Synthesized according to the general procedure, and starting with **6j** and **7c**, this compound was obtained as a yellow solid (138.6 mg, 82% yield). Mp: 116–118 °C; IR (KBr, selected peaks): 3251 (NH), 1735 (C=O), 1590 (C=N), 1491, 1474, 1384, 1315, 1092, 821, 689 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.89 (br s, 1H, NH), 7.78 (d, J = 2.0 Hz, 1H), 7.58–7.50 (m, 3H), 7.41 (d, J = 8.6 Hz, 2H), 7.25–7.19 (m, 2H), 7.15 (t, J = 8.0 Hz, 2H), 7.00–6.92 (m, 3H), 6.91 (d, J = 1.0 Hz, 1H) 6.88–6.83 (m, 1H), 6.80 (t, J = 7.3 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.86 (Cq), 147.07 (Cq), 143.86 (Cq), 142.27 (Cq), 140.24 (Cq), 135.74 (Cq), 135.52 (CH), 134.76 (Cq), 131.32 (CH), 130.30 (2CH), 130.23 (CH), 129.85 (2CH), 129.44 (2CH), 128.98 (Cq), 128.13 (2CH), 127.13 (Cq), 127.03 (CH), 121.15 (CH), 116.21 (Cq), 114.82 (2CH), 113.91 (CH), 88.37 (Cspiro). Anal. Calcd for $C_{27}H_{17}BrCl_2N_4O$: C 57.47, H 3.04, N 9.93, Found: C 57.87, H 2.84, N 9.77.

*4.1.5.21. 5-Bromo-4'-(3-chlorophenyl)-5'-(4-methoxyphenyl)-2′-phenyl-2′,4′-dihydro spiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5u**).* Synthesized according to the general procedure, and starting with **6j** and **7d**, this compound was obtained as a yellow solid (50.2 mg, 60% yield). Mp: 123–125 °C; IR (KBr, selected peaks): 3259 (NH), 1746 (C=O), 1590 (C=N), 1498, 1475, 1385, 1255, 1177, 1032, 689 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.86 (br s, 1H, NH), 7.75 (d, J = 2.0 Hz, 1H), 7.54 (dd, J = 8.3, 2.0 Hz, 1H), 7.47–7.41 (m, 2H), 7.25–7.17 (m, 2H), 7.16–7.09 (m, 2H), 6.96 (d, J = 8.3 Hz, 1H), 6.93–6.87 (m, 5H), 6.85–6.80 (m, 1H), 6.76 (t, J = 7.3 Hz, 1H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.00 (Cq), 161.68 (Cq), 147.92 (Cq), 144.21 (Cq), 142.16 (Cq), 140.64 (Cq), 135.38 (CH), 134.60 (Cq), 131.16 (CH), 130.31 (2CH), 130.16 (CH), 129.78 (2CH), 129.25 (Cq), 128.27 (CH), 127.89 (CH), 127.10 (CH), 120.74 (CH), 120.38 (Cq), 116.19 (Cq), 114.68 (2CH), 114.64 (2CH), 113.80 (CH), 88.10 (Cspiro), 55.67 (CH_3). Anal. Calcd for $C_{28}H_{20}BrClN_4O_2 \cdot 0.3H_2O$: C 59.49, H 3.68, N 9.91, found: C 59.17, H 3.34, N 10.08.

*4.1.5.22. 5-Bromo-2'-(2-chlorophenyl)-4'-(3-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5v**).* Synthesized according to the general procedure, and starting with **6j** and **7e**, this compound was obtained as a white solid (105.2 mg, 63% yield). Mp: 244–246 °C; IR (KBr, selected peaks): 3168 (NH), 1747 (C=O), 1588 (C=N), 1474, 1384, 1196, 828, 761, 690 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.53 (br s, 1H, NH), 7.77 (dd, J = 8.1, 1.5 Hz, 1H), 7.59–7.54 (m, 2H), 7.46–7.30 (m, 5H), 7.22–7.06 (m, 5H), 6.90–6.86 (m, 1H), 6.85–6.81 (m, 1H), 6.77 (d, J = 8.3 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.60 (Cq), 152.48 (Cq), 143.75 (Cq), 143.26 (Cq), 140.86 (Cq), 134.91 (CH), 134.57 (Cq), 131.40 (CH), 131.06 (CH), 130.75 (CH), 130.38 (CH), 129.59 (Cq), 129.24 (2CH), 129.11 (2CH), 128.44 (Cq), 128.31 (CH), 128.10 (CH), 127.96 (CH), 127.63 (CH), 127.26 (CH), 126.99 (Cq), 126.75 (CH), 114.64 (Cq), 113.10 (CH), 89.48 (Cspiro). Anal. Calcd for $C_{27}H_{17}BrCl_2N_4O \cdot 0.55H_2O$: C 56.48, H 3.18, N 9.76, found: C 56.21, H 3.32, N 9.37.

*4.1.5.23. 5-Bromo-2′,4′-bis(3-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro[indoline-3,3'-[1,2,4]triazol]-2-one (**5w**).* Synthesized according to the general procedure, and starting with **6j** and **7f**, this compound was obtained as a yellow solid (147.4 mg, 88% yield). Mp: 118–120 °C; IR (KBr, selected peaks): 3245 (NH), 1735 (C=O), 1589 (C=N), 1474, 1384, 1328, 1197, 773, 691 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 7.83 (d, J = 2.0 Hz, 1H), 7.57 (dd, J = 8.4, 2.0 Hz, 1H), 7.55–7.51 (m, 2H), 7.43–7.35 (m, 3H), 7.23–7.19 (m, 2H), 7.14–7.06 (m, 2H), 6.99 (d, J = 8.4 Hz, 1H), 6.90 (dd, J = 2.4, 1.3 Hz, 1H), 6.88–6.82 (m, 1H), 6.78 (ddd, J = 7.9, 2.0, 0.6 Hz, 1H), 6.65 (ddd, J = 8.3, 2.2, 0.6 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.54 (Cq), 148.91 (Cq), 145.04 (Cq), 142.19 (Cq), 140.09 (Cq), 135.78 (CH), 135.31 (Cq), 134.72 (Cq), 131.30 (2CH), 130.75 (CH), 130.30 (CH), 129.29 (2CH), 128.92 (2CH), 128.39 (Cq), 128.34 (CH), 128.22 (CH), 127.91 (Cq), 127.11 (CH), 120.38 (CH), 116.44 (Cq), 114.56 (CH), 114.00 (CH), 112.25 (CH), 87.88 (Cspiro). Anal. Calcd for $C_{27}H_{17}BrCl_2N_4O \cdot 0.85H_2O$: C 55.95, H 3.26, N 9.67, found: C 55.61, H 3.13, N 9.39.

*4.1.5.24. 5-Bromo-4'-(3-chlorophenyl)-2'-(4-chlorophenyl)-5′-phenyl-2′,4′-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5x**).*

Synthesized according to the general procedure, and starting with **6j** and **7g**, this compound was obtained as a yellow solid (149.4 mg, 89% yield). Mp: 203–205 °C; IR (KBr, selected peaks): 3085 (NH), 1746 (C=O), 1590 (C=N), 1491, 1475, 1385, 1199, 776, 694 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 7.79 (d, J = 2.0 Hz, 1H), 7.58–7.49 (m, 3H), 7.42–7.31 (m, 3H), 7.24–7.17 (m, 2H), 7.15 (d, J = 9.0 Hz, 2H), 6.96 (d, J = 8.2 Hz, 1H), 6.92 (d, J = 9.0 Hz, 2H), 6.89 (dd, J = 2.3, 1.2 Hz, 1H), 6.87–6.80 (m, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.60 (Cq), 148.66 (Cq), 142.87 (Cq), 142.27 (Cq), 140.27 (Cq), 135.65 (CH), 134.70 (Cq), 131.25 (CH), 130.64 (CH), 130.23 (CH), 129.71 (2CH), 129.27 (2CH), 128.85 (2CH), 128.62 (Cq), 128.26 (CH), 128.09 (CH), 128.08 (Cq), 127.02 (CH), 125.23 (Cq), 116.32 (Cq), 116.14 (2CH), 114.02 (CH), 88.18 (Cspiro). Anal. Calcd for $C_{27}H_{17}BrCl_2N_4O \cdot 0.15H_2O$: C 57.19, H 3.08, N 9.88, found: C 56.86, H 3.27, N 9.70.

*4.1.5.25. 5-Bromo-2'-(2-chlorophenyl)-4',5'-bis(3-chlorophenyl)-2',4'-dihydrospiro [indoline-3,3'-[1,2,4]triazol]-2-one (**5y**).* Synthesized according to the general procedure, and starting with **6j** and **7h**, this compound was obtained as a white solid (143.1 mg, 80% yield). Mp: 239–240 °C; IR (KBr, selected peaks): 3242 (NH), 1747 (C=O), 1589 (C=N), 1474, 1431, 1384, 1195, 1077, 764, 695 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.57 (br s, 1H, NH), 7.77 (dd, J = 8.1, 1.5 Hz, 1H), 7.67–7.64 (m, 1H), 7.48–7.30 (m, 5H), 7.24 (d, J = 2.0 Hz, 1H), 7.23–7.14 (m, 3H), 7.11 (ddd, J = 8.0, 7.3, 1.6 Hz, 1H), 6.96–6.92 (m, 1H), 6.91–6.86 (m, 1H), 6.77 (d, J = 8.3 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 173.54 (Cq), 151.10 (Cq), 143.44 (Cq), 143.19 (Cq), 140.53 (Cq), 135.03 (CH), 134.74 (Cq), 134.67 (Cq), 131.53 (CH), 131.24 (CH), 131.00 (CH), 130.65 (CH), 130.49 (Cq), 130.44 (CH), 129.64 (Cq), 128.86 (CH), 128.48 (CH), 128.11 (CH), 128.01 (CH), 127.93 (CH), 127.48 (2CH), 126.82 (CH), 126.67 (Cq), 114.70 (Cq), 113.12 (CH), 89.69 (Cspiro). Anal. Calcd for $C_{27}H_{16}BrCl_3N_4O$: C 54.16, H 2.70, N 9.36, found: C 54.54, H 2.80, N 9.29.

*4.1.5.26. 5-Bromo-2',4',5'-tris(3-chlorophenyl)-2',4'-dihydrospiro [indoline-3,3'-[1,2,4] triazol]-2-one (**5z**).* Synthesized according to the general procedure, and starting with **6j** and **7i**, this compound was obtained as a yellow solid (156.3 mg, 88% yield). Mp: 113–115 °C; IR (KBr, selected peaks): 3240 (NH), 1740 (C=O), 1589 (C=N), 1475, 1430, 1385, 1326, 1195, 768, 691 cm^{-1}.^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 10.02 (br s, 1H, NH), 7.87 (d, J = 2.0 Hz, 1H), 7.64–7.60 (m, 1H), 7.58 (dd, J = 8.3, 2.0 Hz, 1H), 7.46–7.33 (m, 3H), 7.27–7.22 (m, 2H), 7.16–7.08 (m, 2H), 6.99 (d, J = 8.3 Hz, 1H), 6.96 (dd, J = 2.5, 1.3 Hz, 1H), 6.92–6.87 (m, 1H), 6.81 (ddd, J = 8.0, 2.0, 0.8 Hz, 1H), 6.68 (ddd, J = 8.4, 2.3, 0.8 Hz, 1H); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 172.38 (Cq), 147.63 (Cq), 144.79 (Cq), 142.08 (Cq), 139.70 (Cq), 135.87 (CH), 135.34 (Cq), 134.86 (Cq), 134.68 (Cq), 131.43 (CH), 131.34 (CH), 131.01 (CH), 130.61 (CH), 130.41 (CH), 129.90 (Cq), 128.60 (CH), 128.49 (CH), 128.33 (CH), 128.07 (Cq), 127.26 (CH), 127.15 (CH), 120.69 (CH), 116.50 (Cq), 114.65 (CH), 114.00 (CH), 112.39 (CH), 88.05 (Cspiro). Anal. Calcd for $C_{27}H_{16}BrCl_3N_4O$: C 54.16, H 2.70, N 9.36, found: C 54.51, H 2.79, N 9.22.

*4.1.6. Synthesis of 5-bromo-4'-(3-chlorophenyl)-2',5'-diphenyl-2',4'-dihydrospiro [indoline-3,3'-pyrazol]-2-one (**4c**)*

Triethylamine (2.0 equiv, 0.3 mmol, 42 μL) was added dropwise to a mixture of 3-methylene indolin-2-one **13** (1.0 equiv, 0.15 mmol, 50.0 mg), and hydrazonoyl chloride **7a** (2.0 equiv, 0.3 mmol, 69.2 mg) in dry DCM (1.5 mL) under nitrogen atmosphere. The reaction was stirred at room temperature for 5 h. The mixture was then washed with brine (2x) and the aqueous phase extracted with DCM. 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 a gradient of 100% *n*-hexane to *n*-hexane/EtOAc (60:40), and recrystallized from diethyl ether to afford the desired compound.

*4.1.6.1. Compound **4c**.* White solid (59.3 mg, 75%). Mp: 185–187 °C; IR (KBr, selected peaks): 3215 (NH), 1726, 1705, 1596, 1492, 1473, 1384, 1194, 751, 689 cm^{-1}; ^1H NMR (400 MHz, Acetone-d_6) δ (ppm): 9.92 (br s, 1H, NH), 7.81–7.71 (m, 2H), 7.40–7.26 (m, 6H), 7.18–7.07 (m, 4H), 6.98–6.91 (m, 3H), 6.86–6.78 (m, 1H), 6.54 (d, J = 2.0 Hz, 1H), 5.45 (s, 1H, H-4'); ^{13}C NMR (100 MHz, Acetone-d_6) δ (ppm): 176.76 (Cq), 149.40 (Cq), 145.06 (Cq), 141.67 (Cq), 138.24 (Cq), 135.00 (Cq), 133.32 (CH), 132.37 (Cq), 131.17 (CH), 130.05 (CH), 129.89 (CH), 129.84 (CH), 129.74 (2CH), 129.44 (2CH), 129.04 (CH), 128.65 (CH), 128.40 (Cq), 127.55 (2CH), 121.80 (CH), 115.90 (2CH), 114.67 (Cq), 112.95 (CH), 77.22 (Cspiro), 62.34 (CH). Anal. Calcd for $C_{28}H_{19}BrClN_3O \cdot 0.2H_2O$: C 63.16, H 3.68, N 7.89, found: C 62.76, H 4.07, N 7.99.

4.2. Biology: general conditions

4.2.1. Cell culture

HEK 293T (ATCC CRL-11268), MCF-7 (ATCC HTB-22TM) and MDA-MB-231 (ATCC HTB-26TM) cells were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units of penicillin G (sodium salt), 100 mg of streptomycin sulfate and 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA). HCT116 cells were grown in McCoy's 5A supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. CCD18-Co cells were grown in DMEM culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin solution (Sigma-Aldrich), 1% Glutamax (Gibco), 1% Non-Essential Amino Acids Solution (NEAA) (Gibco) and 0.025% TNF-α Human.

4.2.2. Cell treatment

Compound stock solutions were prepared in sterile DMSO. Prior to all treatments, cells were allowed to adhere for 24 h and then exposed to test compounds diluted in culture medium for the indicated time. All experiments were performed in parallel with DMSO vehicle control. The final DMSO concentration did not exceed 0.8% (v/v).

4.2.3. In vitro antiproliferative assays

Cells were seeded in 96-well plates at 2×10^5 cells/mL (MCF-7, MDA-MB-231, HEK 293T), 1×10^5 cells/mL (HCT116), and 4×10^3 cells/well (CCD18-co human normal colon fibroblasts). The cellular growth inhibitory activity was evaluated in five cell lines. Antiproliferative assays in MCF-7, MDA-MB-231, and HEK 293T were assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) [47]. Each compound concentration and DMSO was tested in triplicate in a single experiment which was repeated at least 3 times. Cell viability was assessed 72 h after compound incubation. Briefly, the medium was removed and replaced with fresh medium, and the MTT dye solution was added to each well (5 mg/mL in 10 mM phosphate buffer solution at pH 7.4), and after 3 h of incubations the media was removed and intracellular formazan crystals were solubilized and extracted with DMSO. Absorbance was measured at 570 nm, after 15 min at room temperature using a microplate reader (FLUOstar Omega, BMG Labtech, Germany), and the percentage of viable cells was determined for each compound concentration as described previously [48]. For cytotoxicity assays in HCT116 cells, each compound concentration and DMSO was tested in duplicate in a single experiment

which was repeated at least 3 times. Cell viability was assessed 72 h after compound incubation by using the CellTiter96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI, USA), 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-3a was used as positive control. Additionally, for non-cytotoxic potential assays in CCD18-Co cells, each compound concentration and DMSO was tested in triplicate in a single experiment which was repeated at least 2 times. Cell viability was assessed as described for the HCT116 cell line. The concentrations of the compounds that inhibited cell growth by 50 (IC_{50}) and 80% (IC_{80}) were determined by non-linear regression using GraphPad PRISM software.

4.2.4. Apoptosis evaluation

Apoptosis was quantified using the Guava Nexin Reagent kit (Merck Millipore). The Nexin assay uses two distinct dyes, Annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells, and the cell impermeant dye, 7-AAD, as an indicator of membrane structural integrity. For this purpose, HCT-116 $p53^{(+/+)}$ cells were plated in 24-well plates at 5×10^4 cells/well. Twenty-four hours after plating, cells were exposed to: 1) compounds in test at IC_{50} concentration; 2) compounds in test at 2-fold IC_{50} concentration, and 3) DMSO (control group), for 72 h. After that, the culture medium was collected and cells detached with Accutase. Cells were collected and centrifuged at 500 g for 5 min at 4 °C. The cell pellet was resuspended in PBS/2% FBS. Subsequently, 50 μL of cell suspension were mixed with 50 μL of Guava Nexin reagent and incubated for 20 min, at room temperature in the absence of light. Following the staining procedure, sample acquisition and data analysis of at least 5000 events per sample were performed using the Guava easyCyte™ Flow Cytometer (Merck Millipore) and Nexin software module.

4.2.5. Cell cycle distribution analysis

The effects of compounds on cell cycle progression were determined using a standard propidium iodide (PI) staining procedure followed by flow cytometry analysis. PI is a fluorescent intercalating agent that has high affinity to nucleic acids.

HCT116 $p53^{(+/+)}$cells were plated in 6-well plates at 1.5×10^5 cells/well. Twenty-four hours after plating, cells were treated with the compounds in test at the IC_{50} concentration, or DMSO, for additional 24 or 48 h. After that, cells were detached and collected by centrifugation at 800*g* for 5 min, at 4 °C. Cell pellets were resuspended in cold PBS and added an equal volume of 80% ice-cold ethanol (−20 °C) drop by drop, while vortexing gently. Samples were stored at −4 °C until data acquisition. For cell cycle analysis, cells were centrifuged again at 850 *g* for 5 min, at 4 °C, and cell pellets were resuspended in 25 μg/mL PI (Fluka, Sigma-Aldrich) and 50 μg/mL RNase A (Sigma-Aldrich) and further incubated for 30 min. Sample acquisition and data analysis were performed using the Guava easyCyte™ Flow Cytometer (Merck Millipore) and Guava analysis software, with acquisition of at least 10000 events per sample.

4.2.6. Total protein extraction and quantification

HCT-116 $p53^{(+/+)}$ cells were plated in 60 mm dishes at 8×10^5 cells/dish. Twenty four hours after plating, cells were exposed to compounds in test at the IC_{50} concentration, or vehicle control (DMSO), for 72 h. After that, floating and adherent cells were collected directly in lysis buffer (1% NP-40, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1 mM dithiothreitol, and 1X proteases and phosphatases inhibitors), followed by sonication and centrifugation at 3200*g* for 10 min at 4 °C. Total protein extracts were recovered and stored at −80 °C. Protein concentration was determined by the colorimetric Bradford method using the Bio-Rad Protein Assay reagent (Bio-Rad), according to the manufacturer's instructions. BSA (Sigma-Aldrich) was used as standard, and absorbance measurements were performed at 595 nm using GloMax-Multi + Detection System (Promega).

4.2.7. Caspase-3 and -7 activity assay

Caspases-3 and -7 activity was measured using the Caspase-Glo 3/7 Assay (Promega). Fifteen μg of total protein extracts from MDA-MB-231 cells in a total volume of 50 μL were mixed with 50 μL of caspase-Glo 3/7 reagent with orbital shaking for 30 s. Subsequently, the mixture was incubated at room temperature for 30 min. The luminescence signal was measured using the GloMax-Multi + Detection System (Promega). Data were analyzed using Excel and GraphPad PRISM software. Doxorubicin was used as positive control.

4.2.8. Western blot and densitometric analysis

Steady-state protein levels were determined by Western blot analysis. Briefly, total protein extracts were separated on 8% and 14% (w/v) sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using the *Trans*-blot Turbo Transfer System (Biorad). Uniform protein loading and transfer was confirmed by transient staining with 0.2% Ponceau S (Merck, Darmstadt, Germany). Next, nonspecific binding sites were blocked with a 5% milk solution in Tris-buffered saline (TBS) for 1 h. Membranes were then incubated overnight at 4 °C with mouse monoclonal anti-p53 (Pab-240, sc-99, 1:200) and anti-Mdm2 (SMP-14, sc-965, 1:200) antibodies, (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated with anti-mouse secondary antibody conjugated with horseradish peroxidase (Bio-Rad) for 2 h at room temperature. The immunoreactive complexes were visualized by chemiluminescence with Immobilon™ Western (Milipore) or SuperSignal West Femto substrate (Thermo Fisher Scientific, Waltham, MA, USA). Ponceau S was used as loading control. Densitometric analysis was performed with the Image Lab software Version 5.1 Beta (Bio-Rad).

4.2.9. Venus-based bimolecular fluorescence complementation (BiFC) assay

To evaluate p53-MDM2 protein-protein interaction, HCT116 $p53^{(-/-)}$ cells were co-transfected using 1 μg of each BiFC pair plasmid (V1-p53/MDM2-V2) and Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Four to 6 h after transfection, the medium was replaced with fresh medium, and compound **5y** and nutlin-3a were added to a final concentration of 5 and 10 μM. The same concentrations of DMSO were tested as control. Cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) (Invitrogen), treated with Accutase (Gibco) and harvested in culture medium. Cell suspensions were centrifuged, supernatants discarded, and cell pellets resuspended in PBS supplemented with 2% FBS [34]. Fluorescence was measured using Guava® easyCyte™ Flow Cytometer (Merck Millipore).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.09.037.

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