Effects of Spiro-bisheterocycles on Proliferation and Apoptosis in Human Breast Cancer Cell Lines

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Abstract. Breast cancer is the leading cause of cancer-related death in women worldwide and a critical public health concern. Here we investigated the anticancer potential and effects of low-molecular-weight bridgehead oxygen and nitrogen-containing spiro-bisheterocycles on proliferation and apoptosis of the human breast cancer cell lines MCF-7 and MDA-MB-231. The compounds feature a hydantoin moiety attached to either diazole, isoxazole, diazepine, oxazepine or benzodiazepine via the privileged tetrahedral spiro-linkage. Treatment with compounds spiro [hydantoin-isoxazole] and spiro [hydantoin-oxazepine] resulted in a dose-dependent decrease of cell proliferation and induction of apoptosis in both breast cancer cell lines, whereas spiro [hydantoin-diazepine] was only active against MDA-MB 231. Quantitative reverse transcription polymerase chain reaction analysis showed up-regulation of murine double minute 2 (MDM2), strictly p53-dependent, and detected an increase in expression of pro-apoptotic caspase 3 and BCL2-associated X (BAX) genes in both breast cancer cell lines expressing wild-type and mutant p53. In summary, the results suggest that our compounds promote apoptosis of breast cancer cell lines via p53-dependent and -independent pathways.

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Breast cancer is the most common invasive cancer in women and the leading cause of cancer-related death in women worldwide, making it an urgent public health issue (1). Despite intensive research into new chemotherapeutic agents, treatment is still characterized by unwanted side-effects and the spread of drug resistance, and thus falls short of expectations (2). Consequently, it is essential to pursue the drug development effort to treat breast cancer cells.

Many spiro compounds play fundamental roles in biological processes and have demonstrated therapeutic properties. (3), especially the spiro-heterocyclic compounds, which have shown promising results in chemotherapy of various cancer types (4). Spiro compounds are usually a structural system of two rings positioned orthogonally one to the other due to the sp³ hybridization of the central spiro carbon, which can be chemically adjusted by introducing various heterocyclic motifs mimicking those found in biomolecules, such as DNA and proteins, in order to increase their ability to interact with biological systems. This very rare spatial arrangement of spiro-bicyclic scaffolds is characterized by high rigidity, preventing freely-rotating bonds, which can also be configured with several essential functional groups.

Spiro functionality also remains a primary structural tool for creating powerful molecular antitumor and anticancer agents used in latest-generation chemotherapy. Nature provides outstanding spiro structures, as evidenced by the discovery of new spiro-bicyclic triterpenoid models were found to be cytotoxic against breast human cancer cell lines (5). However, the low natural availability of spiro compounds has prompted researchers to build on these molecular templates to optimize novel spiro-based chemical structures capable of significant bioactivity.

In this context, researchers have successfully used tetrapeptide recognition motifs in order to prepare core systems of 5.5 spiro-oxindoles (Figure 1A) and 6.5.5 spiro-bicyclic lactams (Figure 1B) capable of affecting protein–protein interactions for use as an efficient strategy in cancer therapy (6). Various spiro-isoxazolidine (Figure 1C) derivatives were prepared and tested for their anticancer activity against human cancer cell lines. Moreover, several 4-bromo spiro-isoxazoline structures have also shown antiproliferative effects against breast and prostate cancer cell lines (7).

The transcription factor tumor suppressor p53 is one of the main mediators regulating the cell cycle and induction of apoptosis in response to cellular damage (9). p53 regulation in the cell is essentially based on its negative regulator murine double minute 2 (MDM2). Targeting p53–MDM2 interaction by using appropriate molecules offers an attractive strategy for p53 activation (8) and a promising approach for new anticancer therapies (10).

Previous studies have reported that spiro compounds are potent antagonists of the p53–MDM2 interaction (6). Given these biorelevant properties, spiro compounds make very attractive targets for cancer treatment screening. Here we provide an account of the evaluation of our previously reported small molecules containing oxygen and nitrogen spiro-bisheterocycles in terms of effects on *in vitro* proliferation and apoptosis of human breast cancer cell lines (MCF-7 and MDA-MB231). The wider aim is to identify novel low-molecular-weight, easily-accessible synthetic spiro compounds for cancer chemotherapy.

Materials and Methods

Chemistry. Spiro-bisheterocyclic compounds **2-7** (Table I; Figure 2) were chemically synthesized following our previously described procedure (11). Their preparation is based on the action of bisnucleophiles such as methylhydrazine, hydroxylamine, ethylenediamine, hydroxyethyleneamine and ortho-phenylenediamine on the key spiro[chromanone-hydantoin] (**1**) dyad, which results in a spiro-to-spiro ring transformation of the chromanone residue into new substituted spiro[hydantoin-diazole] (**2**), spiro[hydantoin-isoxazole] (**3**), spiro[hydantoin-diazepine] (**4-5**), spiro[hydantoin-oxazepine] (**6**) and spiro[hydantoin-benzodiazepine] (**7**) (Figure 2). During these chemical reactions, the hydantoin cycle is preserved, as confirmed by relevant 2D nuclear magnetic resonance and single-crystal X-ray diffraction studies. Our spiro-bisheterocycles (**2-7**) were produced in optimal 42-67% yields after chromatographic purification and recrystallization, thus affording high purity as checked by TLC showing a single spot.

Cell culture and treatment. MCF-7 and MDA/MB 231 human breast adenocarcinoma cell lines were purchased from the American Type Culture Collection (Molsheim, France). MCF-7 and MDA-MB-231 were grown in RPMI-1640 and L-15 medium (GIBCO, Invitrogen, Saint-Aubin, France), respectively. RPMI-1640 medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Eurobio, Courtaboeuf, France), 1% L-glutamine (2 mM) (GIBCO,

Table I. Structures and experimental data of spiro-bisheterocycles **2-7** (11).

Compound	Structure	Molecular weight (g/mol)	Yield (%)
2		424.55	61
3		411.50	42
4		438.57	46
5		358.44	67
6		439.56	59
7		406.49	63

Invitrogen, Saint-Aubin, France), 0.5% gentamycin (50 mg/ml) (Fisher Scientific, Strasbourg, France) and 0.05% insulin (100 UI/ml). Fluoresceinisothiocyanate (FITC) conjugated to annexin V was purchased from BioLegend (San Diego, CA, USA) and propidium iodide (PI) was purchased from Biotium (Hayward, CA, USA). A 10 mM stock solution of compounds **2-7** was prepared in dimethylsulfoxide (DMSO) and stored at 4°C. All compounds were added to cells at different concentrations. The DMSO concentration of controls was <0.1% (v/v) and 1 μ M doxorubicin was used as a positive control.

Cell proliferation assay. Cell proliferation was assessed by resazurin assay. Human breast adenocarcinoma cell lines (1×10^4 /well) were cultured in 96-well plates with complete media for 24 h. At the end of incubation, cells were treated with compounds **2-7** at concentrations of 0, 10, 25, 50 and 100 μ M for 72 h at 37°C under a 5% CO₂ atmosphere without replacing the medium. Control cells and positive control cells were treated with 0.1% DMSO and 1 μ M doxorubicin, respectively. After incubation, 200 μ l of a 25 μ g/ml solution of resazurin in medium was added to each well. Plates were incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO₂. Fluorescence was then measured on an automated 96-well plate reader (Fluorskan Ascent FL; Thermo Fisher Scientific, Wilmington, NC, USA) at 530 nm excitation wavelength and 590 nm emission wavelength. Under these conditions, fluorescence (OD value) was proportional to the number of living cells in the well. Cell

Figure 1. Synthetic spiro-bicyclic compounds as anticancer agents: A: 5.5 spiro-oxindoles; B: 6.5.5 spiro-bicyclic lactams; and C: spiro-isoxazolidine.

Figure 2. Reaction pathway for the synthesis of spiro-bisheterocycles 2-7 (11). RT: Room temperature; DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; THF: tetrahydrofuran.

proliferation assays were performed at least six times for each cell line (in replicates of six wells per concentration tested) (12).

DNA quantification using Hoechst 33342. 96-Well plates containing treated and control cells were taken out of freezer storage at -20°C and left to defrost for 1 h at room temperature. Then, 100 μl of 0.01% sodium dodecyl sulfate was added to enable the DNA to come into contact with the Hoechst solution. After 1 h of incubation, plates were put back in the freezer at -80°C for 1 h to trigger thermal shock. After defrosting for 2 h, 100 μl of Hoechst solution 33342 (30 $\mu\text{g ml}^{-1}$) was added to each well. Plates were

placed under agitation for 1 h shielded from light. The reading of the fluorescence was made by Fluorskan Ascent FL (Thermo Fisher Scientific). Hoechst 33342 dye is a fluorescent nucleic acid which exhibits a maximum emission at 460 nm when bound specifically to double-stranded DNA. Data represent viable cells.

Annexin V-FITC/PI apoptosis assay. MCF-7 and MDA-MB 231 tumor cells (10^5 cells/well) were treated with 100 μM of compounds 3, 4 and 6 for 72 h at 37°C and with 5% CO_2 , then washed with phosphate-buffered saline, recovered by centrifugation at $1,000 \times g$ for 5 min at room temperature (RT; 25°C), and resuspended in 40 μl

Table II. *Primer sequences.*

Target gene	Name	Primer sequences
<i>p53</i>	Tumor protein p53	F: TCAGTCTACCTCCCGCCATA R: TTACATCTCCCAAACATCCCT
<i>MDM2</i>	Murine double minute 2	F: CATTGAACCTTGTGTGATTTGTC R: GCAGGGCTTATTCCTTTTCTTTA
<i>BAX</i>	BCL2-associated X	F: CCTGTGCACCAAGGTGCCGGAAC R: CCACCCCTGGTCTTGGATCCAGCCC
<i>CASP3</i>	Caspase-3	F: ACATGGCGTGTCAAAAATACC R: CACAAAGCGACTGGATGAAC
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	F: CACATGGCCTCCAAGGAGTAA R: TGAGGGTCTCTCTCTCCTCTTGT

of annexin V binding buffer (140 mM NaCl, 10 mM HEPES/NaOH, 2.5 mM CaCl₂). The suspension was stained with 5 µl of annexin V-FITC and 5 µl of PI, then incubated for 15 min at RT in the dark. To the sample was added 250 µl of 1xphosphate-buffered saline, this was then centrifuged at 1,000 × *g* for 5 min at RT, and cells were resuspended in 50 µl of annexin V binding buffer. Stained cells were then analyzed on a Cellometer K2 image cytometer (Nexcelom Bioscience, Lawrence, MA, USA).

Isolation of RNA and real-time polymerase chain reaction. Total RNA was extracted from untreated and treated breast cancer cell lines, MCF-7 and MDA-MB-231, using Trizol and quantified on a NanoDrop spectrophotometer (Nanodrop 2000; Thermo Scientific, Waltham, MA, USA). Purity was estimated by 260/280 nm absorbance ratio. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France). Quantitative real-time PCR analysis was performed using a SYBR Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France) as follows: first step at 50°C for 2 min, denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min. In total, 10 ng of cDNA was added to 18 µl of reaction containing primers. Relative expression levels of *p53*, *MDM2* and BCL2-associated X (*BAX*) were calculated using the 2^{-ΔΔCt} method. Experiments were repeated in triplicate. The primer sequences used are listed in Table II.

Data analysis. Data are expressed as the mean±SEM of multiple experiments. Statistical significance was determined by Student's *t*-test using SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA).

Results

Effects of spiro-bisheterocycles 2-7 on MCF-7 and MDA-MB231 cell proliferation. In order to determine the impact of spiro-bisheterocycles 2-7 on cell proliferation of human breast cancer cell lines, MCF-7 and MDA-MB 231 cells were treated with compounds 2-7 at different concentrations from 10 to 100 µM for 72 h. Cell proliferation was assessed after 2 h of incubation with resazurin solution. Proliferation of MCF-7 human breast cancer cell line was significantly reduced with compounds 3 and 6, with The half-maximal inhibitory concentration (IC₅₀) corresponding to 42.3 µM (17.4 µg/ml) and 66.3 µM (30.8 µg/ml) (Figure 3), respectively. We also observed

significant inhibition of MDA-MB-231 cells (Figure 3) up to 67.9 µM (28.5 µg/ml) for 3 and 97.1 µM (42.7 µg/ml) for 6. Proliferation of MDA-MB 231 cells was also significantly reduced with compound 4, with IC₅₀ of 44.6 µM (19.6 µg/ml), but not of MCF-7 cells. On the other hand, treatment with compounds 2, 5 and 7 did not induce a significant decrease in cell proliferation in the two breast cancer cell lines. At a 100 µM, compound 3 was the most effective, inducing a major suppressive effect of 98.15% and 97.02% against MCF-7 and MDA-MB 231 cells, respectively.

Hoechst staining confirmed results of the resazurin assay and showed significant impact of compounds 3 and 6 on MCF-7 and MDA-MB-231 tumor cell proliferation (Figure 4), with similar IC₅₀. Compound 4 induced tumor inhibition at an IC₅₀ of 42 µM inMDA-MB-231 cells.

Effects of spiro-bisheterocycles 3, 4 and 6 on apoptosis. In order to determine the apoptotic efficacy of the spiro-bisheterocycles, cells were treated with 100 µM of 3, 4 and 6 for 72 h (Figure 5), then collected and subjected to Cellometer annexin V-FITC/PI apoptosis assay. Annexin V-FITC⁻/PI⁻ flagged viable cells, annexin V-FITC⁺/PI⁻ flagged early-stage apoptotic cells, and annexin V-FITC⁺/PI⁺ flagged late-stage apoptotic cells.

Analysis showed a sharp induction of apoptosis by compounds 3 and 6 in MCF-7 cells and by 3, 4 and 6 in MDA-MB-231 cells (Figure 5A). The rates of early and late apoptosis were 9.5%, 85.4% and 71.3% in MCF-7 for control, and compounds 3 and 6, respectively, and 5.9%, 88.1%, 79.4% and 66.4% in MDA-MB-231 cells for control, and compounds 3, 4 and 6, respectively (Figure 5B).

Effects of spiro-bisheterocycles 3, 4 and 6 on p53, MDM2, BAX and caspase 3 expression. In an effort to investigate whether spiro compounds 3, 4 and 6 induce apoptosis of breast cancer cell lines by targeting p53–MDM2 interaction, we used quantitative reverse transcription-polymerase chain reaction to analyze the wild-type *p53*, *MDM2*, *BAX* and

Figure 3. Effect of compounds 2-7 on the *in vitro* growth and proliferation of human breast cancer cells. MCF-7 and MDA-MB231 cell line were either untreated, treated with 1 μ M doxorubicin (Dox) or treated with 10, 25, 50 and 100 μ M of compounds 2-7 for 72 h as described in the Materials and Methods section. Proliferation was assessed using resazurin on an automated 96-well Fluoroskan Ascent FL. Columns show means $\pm$ SD, n=6. *Significantly different at $p<0.05$.

caspase 3 gene expression levels. Results for MCF-7 breast cancer cells show that compound 3 more clearly increased the expression of wild-type p53 (6.707-fold vs. control) than did compound 6 (3.275-fold vs. control) (Figure 6). We also found

that compounds 3 and 6 induced up-regulation of *MDM2* in MCF-7 cells at levels of 4.282 and 2.658, respectively. However, in MDA-MB-231 p53-mutant breast cancer cells, no significant changes in the levels of *p53* and *MDM2* were

Figure 4. Inhibitive effect of compounds 3, 4 and 6 on growth of cancer cell lines MCF-7 and MDA-MB-231. *Significantly different at $p < 0.05$.

observed with compounds 3, 4 and 6, indicating that overexpression of *MDM2* is under control of *p53*. Compared to levels in untreated control MCF-7 cells, compounds 3 and 6 led to 6.738- and 3.532-fold higher *BAX* and 6.498- and 2.703-fold higher caspase 3 gene expression, respectively. Interestingly, similar results were obtained in MDA-MB-231 cells, where an increase of the expression of caspase 3 and *BAX* genes was also noted with compounds 3, 6 and, even with compound 4. This demonstrates the capacity of our compounds to induce apoptosis independently of *p53* status.

Discussion

Tumor suppressor *p53* plays a key role in the regulation of cell cycle and apoptosis (13-15). We previously reported that *p53* inhibition by its negative regulator *MDM2* is involved in the onset of breast cancer (16, 17). An approach based on restoring *p53* activity by inhibiting *p53*-*MDM2* interaction could lead to tumor suppression and thus be a promising strategy for future control of breast cancer (18, 19). Many spiro compounds possess very promising biological and pharmacological activities, especially antitumor and antimicrobial properties (4, 20, 21).

These specific, potent, non-peptide small-molecule inhibitors are reported to mimic the α -helix recognition motif of the *p53*-

MDM2 complex (22), thereby efficiently reactivating *p53* tumor-suppressor activity and consequently inducing a *p53*-mediated signaling pathway that will culminate in cell death by apoptosis (23). Our results clearly showed that the spiro-heterocyclic compounds substituted by isoxazole (3), diazepine (4) and oxazepine (6) significantly reduced the proliferation of MCF-7 and MDA-MB-231 cells in a dose-dependent manner. These results were consistent with other studies demonstrating the cytotoxicity of spiro-bicyclic compounds in human cancer cell lines (5, 24). Our data are further supported by another report which demonstrated similar *in vivo* anticancer potential of a spiro-heterocyclic compound on the development on MCF-7 human breast cancer cells (25). The antiproliferative effect was more pronounced with spiro-isoxazole (3). Structurally, the presence of the isoxazole heterocyclic unit is likely tightly related to these clear antiproliferative effects, as already reported for other spiro-models containing the isoxazole motif (7). It was also observed that the presence of oxazepine substituent demonstrated good activity against breast cancer cells (26), thus explaining the antiproliferative effect observed with compound 6. Furthermore, the significant antiproliferative activity of compound 4 could stem from the presence of a diazepine molecule in the structure, since diazepine inhibits the adenosine A2B receptor involved in the development of tumors and proliferation (27). In addition, a

Figure 5. Drug-induced apoptosis in breast cancer cell lines MCF-7 and MDA-MB-231 at 72 h of incubation with spiro-bisheterocyclic compounds. A: Bright-field (top row), and fluorescent images of annexin V-fluorescein isothiocyanate (FITC) (middle row) and propidium iodide (PI) (bottom row) staining. The images show significant induction of apoptosis with compounds 3 and 6 in MCF-7 cells, while strong apoptosis was observed with compounds 3, 4 and 6 in MDA-MB-231 cells. B: Quantitative results of percentage of viable, and early apoptotic and late apoptotic cells after treatment with compounds. *Significantly different at $p < 0.05$.

previous study reported that adenosine A_{2B} receptors are expressed at high levels in the estrogen-negative MDA-MB-231 cell line but not expressed in the estrogen-positive MCF-7 cell line, which could explain why the antiproliferative effect of compound 4 was observed here only in MDA-MB-231 cells (28). Moreover, the presence of cyclohexyl fragments in these three compounds might also contribute to their antiproliferative activity (29), allowing better understanding of why compounds 5 and 7 had no effect.

Apoptosis plays a key role in the maintenance of homeostasis (30) and is considered a primary form of cancer cell death (31). This pathway results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, and finally uptake by phagocytic cells (32). Recent reports indicate that most spiro compounds induce apoptosis of cancer cells (33, 34), but especially spiro-isoxazolidine derivatives (35). Here, we performed annexin V-

FITC/PI apoptosis assays and demonstrated that our spiro-bisheterocyclic compounds induced apoptosis of breast cancer cell lines. Annexin V is a protein that interacts strongly and specifically with phosphatidylserine. During early apoptosis, phosphatidylserine translocates to the external leaflet and can be used for the detection of apoptosis (36). Analysis using Cellometer K2 image cytometry indicated that compounds 3, 4 and 6 induced a large proportion of breast cancer cell death by apoptosis *in vitro*. The strong effect was observed with compound 3 in both breast cancer cell lines.

As previously demonstrated (18), the most likely mechanism by which spiro compounds initiate apoptosis of wild-type $p53$ breast cancer cells is by inhibition and disruption of the $p53$ -MDM2 interaction, leading to accumulation of $p53$ and up-regulation of $MDM2$. The tumor suppressor $p53$ binds to DNA and mediates transcriptional activation which ultimately overexpress BAX and promote

Figure 6. Results of polymerase chain reaction detection. *p53*, murine double minute 2 (*MDM2*), *BCL2*-associated X (*BAX*) and caspase-3 genes expression in MCF-7 and MDA-MB-231 cells following treatment with 100 μ M of compounds 3, 4 and 6 for 72 h. *Significantly different at $p < 0.05$.

p53-dependent cell apoptosis (37, 38). The pro-apoptotic BAX protein is one of the most important regulators of the intrinsic pathway of apoptosis (39). Excess of BAX protein leads to formation of BAX-BAX homodimers, that stimulate release of cytochrome *c* from mitochondria and activate caspase-3, then inducing apoptosis (40).

Quantitative reverse transcription-polymerase chain reaction data support these findings and showed an increase of wild-type *p53* expression in MCF-7 cells, accompanied by up-regulation of *MDM2*, with treatment of compound 3 and 6. We extended this study by demonstrating that spiro compounds 3 and 6 enhance expression of caspase-3 and

Figure 7. Proposed mechanisms of apoptosis of breast cancer MCF-7 and MDA-MB-231 cells induced by spiro-compounds 3, 4 and 6.

BAX in MCF-7 wild-type *p53* breast cancer cells, and consequently induce apoptosis. At the same time, in order to determine whether our spiro compounds induce apoptosis regardless of cell *p53* status, we also employed MDA-MB-231 cells which harbor mutant *p53*. Treatment with compounds 3, 4 and 6 had no significant effect on expression of *p53* or *MDM2* in cancer cells lacking wild-type *p53* but increased caspase-3 and *BAX* expression. These results correlate with a previous study revealing induction of apoptosis through *p53*-independent pathways (41).

In summary (Figure 7), this study brings the first demonstration that new small spiro-bisheterocyclic molecules can sensitize breast cancer cells to apoptosis by targeting *p53*–*MDM2* interaction. We also highlighted that these spiro compounds promote apoptosis *via* *p53*-independent pathway(s), suggesting that these compounds represent interesting candidates as therapeutic targets for the treatment of breast cancer.

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