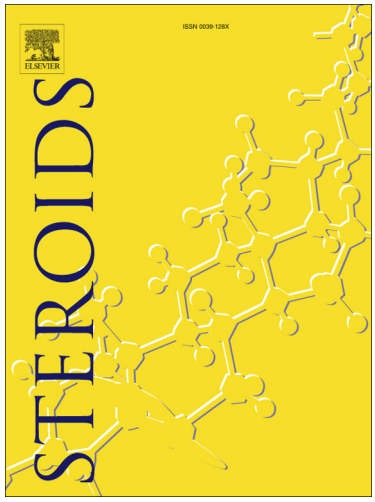

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Efficient synthesis of novel antiproliferative steroidal spirooxindoles via the [3+2] cycloaddition reactions of azomethine ylides

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Abstract: A series of novel steroidal spirooxindoles **3a-h** were synthesized from pregnenolone in a high regioselective manner using the 1,3-dipolar cycloaddition as the key step. This protocol resulted in the formation of two C-C bonds, one C-N bond and the creation of one pyrrolidine ring and three contiguous stereocenters in a single operation. Biological evaluation showed that these synthesized steroidal spirooxindoles exhibited moderate to good antiproliferative activities against the tested cell lines and some of them were more potent than 5-FU. Among them, compounds **3e** and **3f** displayed the best antiproliferative activity against MCF-7 cells with the IC_{50} values of 4.0 and 3.9 μM, respectively. Flow cytometry analysis demonstrated that compound **3d** caused the cellular apoptosis and cell cycle arrest at G2/M phase in a concentration-dependent manner. Docking results indicated that compound **3d** fitted well into the MDM2 active site 1RV1 by interacting with Lys94 and Thr101 residues.

Keywords: Pregnenolone; Spirooxindoles; Antiproliferative activity; Apoptosis; Cell cycle arrest; Docking studies

1. Introduction

Steroids, an important class of polycyclic compounds, have been proved to possess diverse biological activities. [1] Chemical modifications on the steroid nucleus and side chains have been recognized as efficient strategies to access biologically important molecules. Particularly, the incorporation of heterocycles into the steroid scaffolds has always been an intriguing topic in the field of steroids, [2] because the introduction of biologically relevant heterocycles always endows these new hybrids with novel properties or improved potency. [3] For example, *N*-heterocycle-containing abiraterone has been used for the treatment of advanced prostate cancer in clinic [4] while galeterone has successfully finished its phase □ clinical development. [5] Among these heterocycles, spirooxindoles (Fig. 1) have drawn wide attention in consequence of their unique structural scaffolds that are prevalent in numerous naturally occurring oxindole alkaloids [6] and in synthetic molecules with promising biological activities. [7] Three representative drug candidates CFI-400945, [8] SAR405838 [9] and NITD609, [10]

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have entered clinical trials for the treatment of human cancers and malaria, respectively (Fig. 1). Therefore, the introduction of spirooxindole skeleton into the steroid core would be an interesting strategy to construct compound library with structural novelty and biological potential.

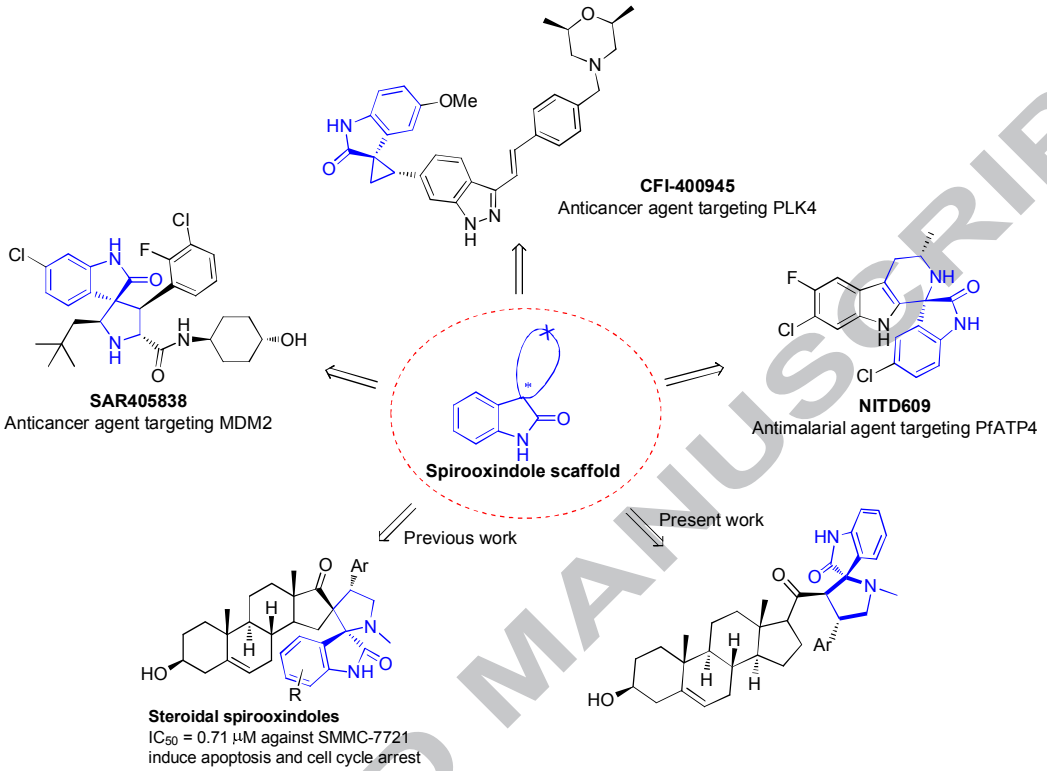


Fig. 1 Spirooxindole-containing biologically active molecules

The broad and promising biological activities of spirooxindole-containing molecules together with intriguing structural architectures have been a major driving force for the development of new synthetic approaches, especially the asymmetric synthesis, [11] as some diastereoisomer always represents markedly enhanced activity compared to its racemates or other diastereoisomers as shown by CFI-400945, SAR405838 and NITD609. The 1,3-dipolar cycloaddition reactions between azomethine ylides and dipolarophiles have been recognized as efficient strategies for the synthesis of pyrrolidines with high yields and stereoselectivity. [12] Very recently, we reported that steroidal spirooxindoles (Fig. 1) can potently inhibit the proliferation of several human cancer cells (IC_{50} = 0.71 μM against SMMC-7721 cells) and induce apoptosis and cell cycle arrest at G2/M phase in a time-/concentration-dependent manner. [13] As part of our efforts toward the identification of novel heterocycles with anticancer potential, [14] we herein report the synthesis of a new series of steroidal spirooxindoles from pregnenolone using the 1,3-cycloaddition reaction as the key step and their *in vitro* antiproliferative activities against several human cancer cell lines. Besides, we also investigated the effects toward the cell cycle and apoptosis. Docking studies were also performed to illustrate the possible binding model of MDM2.

2. Results and discussion

2.1. Chemistry

As shown in Fig. 2, our strategy involved the 1,3-dipolar cycloaddition between 21-arylidenepregnenolone derivatives (**2a-h**) and azomethine ylide generated *in situ* from isatin and sarcosine.

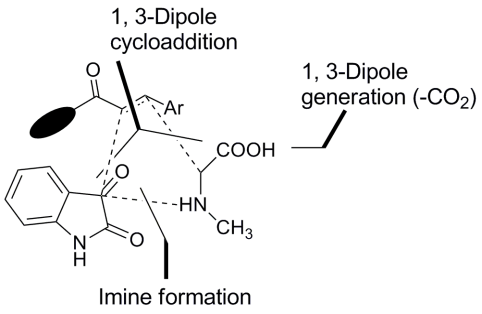
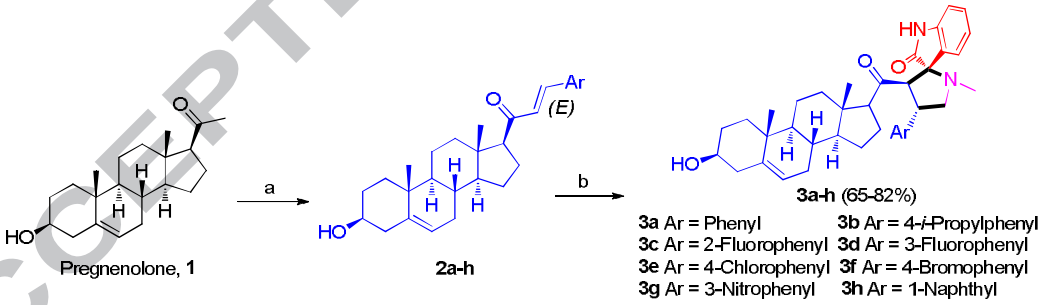


Fig. 2 Strategy employed for the synthesis of steroidal spirooxindoles

21-arylidenepregnenolone derivatives **2a-h** were initially synthesized from pregnenolone and the corresponding aromatic aldehydes in excellent yields following our previously reported method, [13] which were then subjected to the 1,3-dipolar cycloaddition reactions with azomethine ylide generated from the decarboxylative condensation of isatin and sarcosine, affording steroidal spirooxindoles **3a-h** in moderate to good yields (Scheme 1). During optimization of the reaction condition, the mixture of 1,4-dioxane and MeOH (1/1) was found to be the best solvent system for this key transformation. Excess of isatin and sarcosine were necessary for complete conversion of 21-arylidenepregnenolone derivatives **2a-h**. With this optimized condition in hand, we further investigated the scope and reproducibility of this methodology.



Scheme 1 Synthesis of steroidal spirooxindoles. Reagents and conditions: (a) Aromatic aldehyde, KF/Al_2O_3, EtOH, reflux; (b) Isatin, sarcosine, 1,4-dioxane/MeOH (1/1), reflux.

All final compounds **3a-h** were fully characterized by NMR and high-resolution mass spectra (HRMS) as described for compound **3g**. Three singlets at 2.21, 0.89 and 0.12 ppm correspond the peaks of *N*-CH_3 of the pyrrolidine ring, 19-CH_3 and 18-CH_3, respectively. The multiplet at 3.46-3.54 ppm is assigned to the 3α-H of steroid A-ring; The proton attached to C-6 appears as a doublet at 5.23 ppm with a coupling constant of 4 Hz. The broad peak at 2.72 ppm is assigned to H-17 when compared to the ^{1}H

NMR spectrum of pregnenolone. The peak of the *N*-H proton at the oxindole ring appears as a singlet at 8.80 ppm (no direct correlation with any carbon is observed in the HSQC spectrum). The aromatic protons appear as doublets and multiplets at 6.93-8.39 ppm. It is evident from the ^{13}C NMR spectrum of compound **3g** that the peaks of C-20 and carbonyl carbon of oxindole appear at 207.09 and 180.29 ppm, respectively. From the HSQC spectrum, a secondary carbon at 59.95 ppm has direct correlations with two protons at around 3.61 and 3.41 ppm, respectively. Besides, correlations between the methylenic protons (H-3') and the carbon of *N*-CH_3 are also observed from the HMBC spectrum (correlations between methylenic protons and protons of *N*-CH_3 are also observed in the H-H COSY spectrum). All these indicate that the peak at 59.95 ppm can be assigned to the methylenic carbon (C-3') of the pyrrolidine ring. From the HMBC spectrum, a doublet at 3.76 ppm has correlations with C-20, amide carbon and a quaternary carbon at 72.56 ppm, indicating that this doublet is the peak of H-1' and the quaternary carbon is the spiro carbon (C-4'). The proton with the peak at 4.42-4.49 ppm as a multiplet has correlations with H-1' and H-3' from the H-H COSY spectrum, this means the multiplet is the peak of benzylic proton (H-2'). These signal assignments are consistent with the H-H COSY correlations depicted in Fig. 3 (please see the supporting information for 2D spectra of compound **3g**). The presence of a molecular ion peak at *m/z* = 624.3441 ($[M+H]^+$) in the mass spectrum (calcd. 624.3437) further confirms the structure of **3g**. The crystal structures of steroidal spirooxindoles synthesized using a similar method have been recently reported. [15]

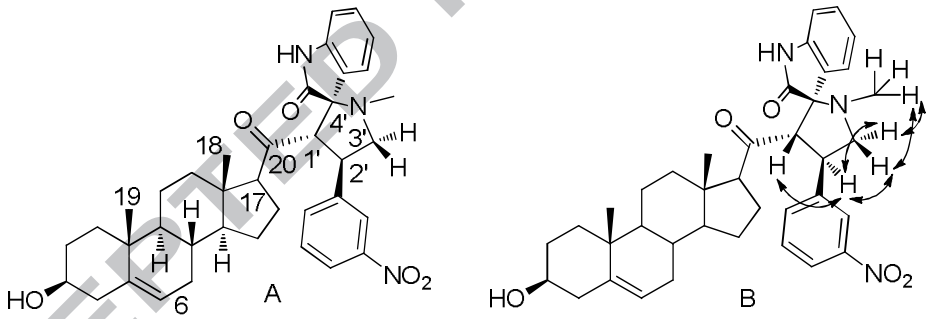
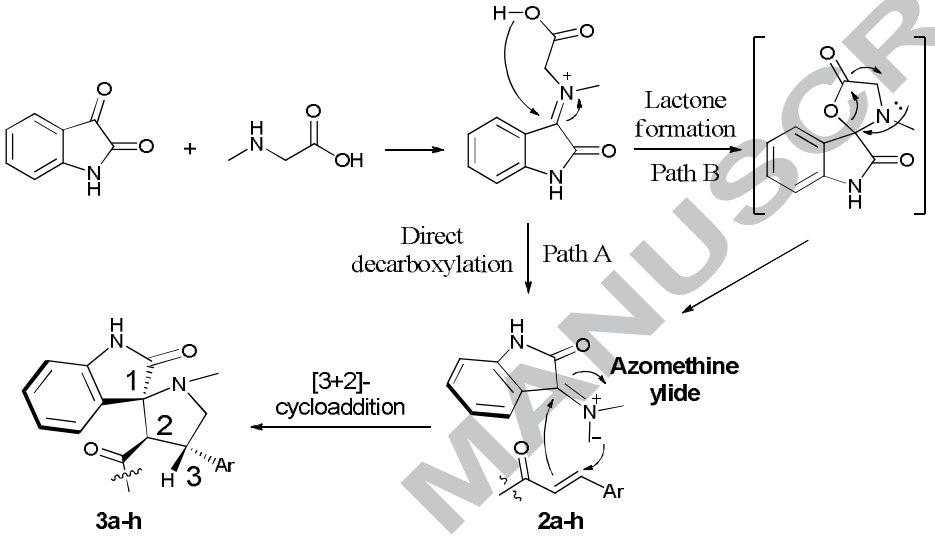


Fig. 3 Selected H–H COSY correlations of compound **3g** (arbitrary numbering)

The possible mechanism for the formation of steroidal spirooxindoles is outlined in Scheme 2. The key azomethine ylide intermediate was formed through the direct decarboxylation of iminium salt formed from isatin and sarcosine (Path A) or the formation of lactone intermediate (Path B). The azomethine ylide then underwent 1,3-dipolar cycloaddition reactions with dipolarophiles **2a-h** in a regioselective manner. The regioselectivity of the reaction is explicable by the preference of the electron-rich carbon of the dipole adding to the electron-deficient β-carbon of the α,β-unsaturated moiety of **2a-h**. The steroidal spirooxindoles were also formed with high stereoselectivity although three stereocenters were formed. The carbonyl group linked to the steroid ring system and the amide carbonyl are in trans-relationship with respect to C_1-C_2 bond of the pyrrolidine ring system, this is presumably ascribable to

the preferred spatial arrangement of the dipolarophile and the azomethine ylide in the cycloaddition step, which would minimize the repulsion between the carbonyl groups. The aryl ring attached to the C-3 position is also the trans-relationship to the carbonyl of the steroid skeleton with respect to C_2-C_3 bond of the pyrrolidine ring system due to the *E*-configuration of the double bond of 21-arylidenepregnenolone derivatives **2a-h**. Besides, it should be noted that three continuous stereocenters (one quaternary carbon centers), two C-C bonds and one C-N bond were formed in one-pot procedure under catalyst-free condition. The high regioselectivity and stereoselectivity were also observed in our previous work [13] and by other groups. [16]



Scheme 2 The possible mechanism for the formation of steroidal spirooxindoles.

2.2. Biological evaluation

2.2.1. Antiproliferative activity

In our previous work, [13] we found that steroidal spirooxindoles showed moderate to good inhibitory activity against EC109, MGC-803, SMMC-7721 and MCF-7 cell lines. Structure-activity relationships (SARs) studies revealed that substituents attached to aryl groups (Fig. 1) played an important role in the activity. To explore the SARs further, we designed and synthesized another new series of steroidal spirooxindoles (**3a-h**) from pregnenolone by varying the steroid nucleus and the aryl groups compared to our previous work. Steroidal spirooxindoles **3a-h** were then subjected to antiproliferative evaluation against four human cancer cell lines (T24, SMMC-7721, MCF-7 and MGC-803) using the MTT assay. The well-known anticancer drug 5-fluorouracil (5-FU) works principally through irreversible inhibition of thymidylate synthase, while some steroidal derivatives such as estradiol, progesterone, DHEA, testosterone could also markedly inhibit thymidylate synthase. [17] Due to the similar mode of action, 5-FU was used as the positive control in the antiproliferative assay.

Table 1 *In vitro* antiproliferative activity against four human cancer cell lines

No	Ar	IC_{50} (μM) [a]			
		T24	SMMC-7721	MCF-7	MGC-803
3a	Phenyl	23.3±1.4	18.8±1.3	5.1±0.7	8.3±0.9
3b	4-*i*-Propylphenyl	80.9±1.9	35.2±1.6	21.8±1.3	15.6±1.2
3c	2-Fluorophenyl	4.1±0.6	43.1±1.6	17.7±1.3	16.1±1.2
3d	3-Fluorophenyl	6.1±0.8	6.7±0.8	4.6±0.7	10.2±1.0
3e	4-Chlorophenyl	15.4±1.2	18.5±1.3	4.0±0.6	6.2±0.8
3f	4-Bromophenyl	14.4±1.2	9.3±1.0	3.9±0.6	11.0±1.0
3g	3-Nitrophenyl	8.8±1.0	44.0±1.6	10.2±1.1	13.5±1.1
3h	1-Naphthyl	44.1±1.6	12.2±1.1	9.9±1.0	14.0±1.2
5-FU	---	7.1±0.9	4.3±0.6	10.5±1.6	9.1±1.0

[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are shown as the means ± SDs of three independent experiments.

As shown in Table 1, all the synthesized compounds showed moderate to good antiproliferative activities against four human cancer cell lines with IC_{50} values ranging from 3.9 to 80.9 μM and some of them were more potent than 5-FU. The SARs were probed by altering the substituents on the phenyl ring. It is evident that the electronic nature of substituents on the phenyl ring had remarkable effect on the activity. For T24 cells, compounds **3a**, **3b** and **3h** with electron-rich aromatic ring represented markedly decreased inhibitory effect than those with electron-deficient substituents (**3c-g**). Compounds **3c** and **3d** with a fluoro atom showed the best inhibition against T24 cells with the IC_{50} values of 4.1 and 6.1 μM, respectively and were slightly more potent than 5-FU. By contrast, all the compounds showed moderate antiproliferative activity against SMMC-7721 cells. However, none of them had better inhibition than 5-FU. Intriguingly, most of these compounds were more active than 5-FU against MCF-7 cells. Among them, compounds **3e** and **3f** with a chloro and bromo atom had the best but comparable activity with the IC_{50} values of 4.0 and 3.9 μM, respectively. Compound **3d** with a fluoro atom at the 3-position (IC_{50} = 4.6 μM) was more than 3-fold potent than compound **3c** with a fluoro atom at the 2 position. It is evident that compounds with an elecron-withdrawing group were generally more potent than those having an elecron-donating group toward MCF-7 cells. Besides, the moderate inhibitory effect was observed for the tested compounds against MGC-803 cells. However, it should be noted that all the compounds had comparable antiproliferative activity with 5-FU regardless of the electronic nature of their substituents. Inspired by this finding, the synthesis of more analogs and further investigation of modes of action are in progress in our group.

2.2.2. Apoptosis assay

Considering the moderate to good antiproliferative activities of these compounds against the tested human cancer cell lines, compound **3d** was chosen to further explore its mechanism of action. In order to characterize the mode of cell death

induced by compound **3d**, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and annexin-V-FITC in MCF-7 cells. After treatment MCF-7 cells with compound **3d** for 24h at different concentrations (0, 2.0, 4.0, 8.0 μM), MCF-7 cells were labeled with the two dyes, and the resulting red (PI) and green fluorescence (FITC) was monitored by flow cytometry. As shown in Fig. 4, compound **3d** caused the remarkable early apoptosis in a concentration-dependent manner. The apoptosis rate was 4.8% for the control group, while the rate was increased to 21.4% at the concentration of 8.0 μM.

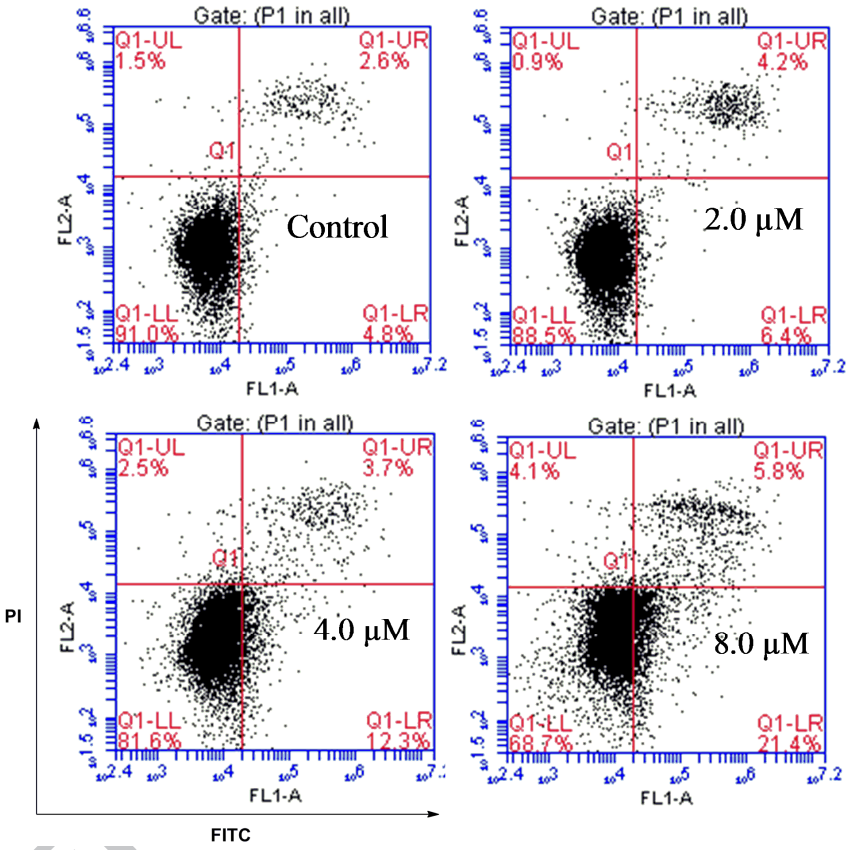


Fig. 4 Apoptotic effect of compound **3d** toward MCF-7 cells. Apoptotic cells were detected with Annexin V-FITC/PI double staining after incubation with compound **3d** at different concentrations (0, 2.0, 4.0, 8.0 μM) for 24 h. The lower left quadrants represent live cells, the lower right quadrants are for early/primary apoptotic cells, upper right quadrants are for late/secondary apoptotic cells, while the upper left quadrants represent cells damaged during the procedure.The experiments were performed three times, and a representative experiment is shown.

2.2.3. Cell cycle analysis

Molecules that inhibit the growth of cancer cells invariably cause alteration of cell cycle distribution, with preferential G2/M blockade. To better understand the antiproliferative activity of compound **3d**, a cell cycle progression was performed by treating MCF-7 cells at different concentrations of compound **3d** (0, 2.0, 4.0, 8.0 μM).

As shown in Fig. 5, when treating MCF-7 cells for 12 h, the percentage of cells at G2/M phase was increased from 18.9% of the control sample to 45.4% at the highest concentration of **3d**. The result revealed that **3d** caused an obvious G2/M arrest in a concentration-dependent manner with a concomitant decrease of cells in other phases of the cell cycle.

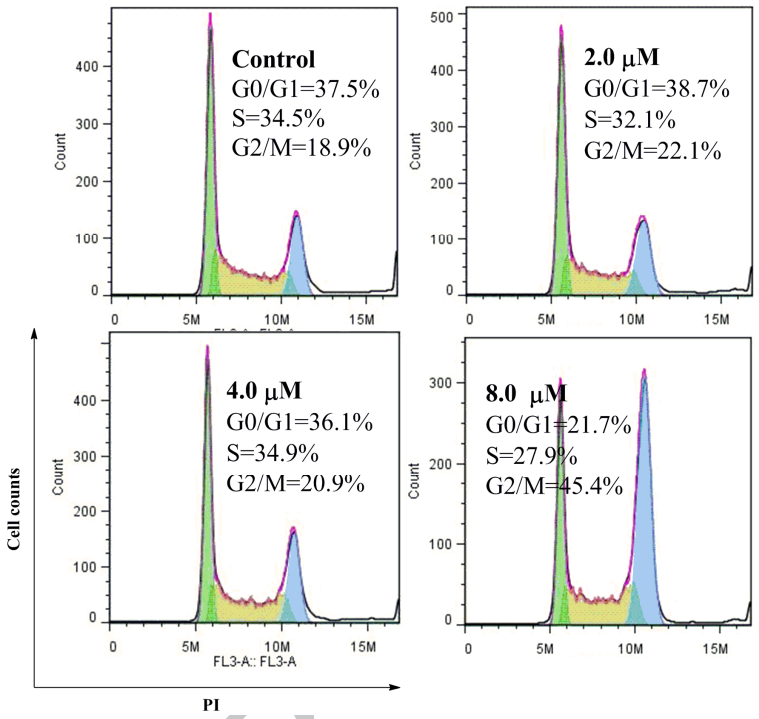


Fig. 5 Effect of compound **3d** on the cell cycle distribution of MCF-7 cells. Cells were treated at different concentrations (0, 2.0, 4.0, 8.0 μM) for 12 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. The experiments were performed three times, and a representative experiment is shown.

2.3.3 Docking studies

Spirooxindoles have been reported to be able to potently inhibit MDM2-p53 interaction, extensive modifications conducted by Wang's group yielded anticancer drug candidate SAR405838 (also known as MI-77301, K_i = 0.88 nmol/L) [9] and its second-generation MI-1061 (K_i = 0.16 nmol/L). [18] Inspired by this interesting finding and considering that our structures share the same spiro-pyrrolidinyl oxindole scaffold with SAR405838 and MI-1061, the docking simulations in the active sites of MDM2 were performed by the AutoDock tools and Autodock Vina program. [19] The docking results would help us visualize the potential receptor-ligand interactions of steroidal spirooxindoles with MDM2 and guide further SAR studies. The crystal structure of MDM2 (PDB: 1RV1) was extracted from RCSB Protein Data Bank; the target protein structure of 1RV1 was docked with the most active compound **3d.** The lowest energy pose of compound **3d** at MDM2's active site is shown in Fig. 6. Compound **3d** fitted well into this pocket showing two hydrogen binding interactions with Lys94 and Thr101 residues. The F-H and N-O distance are 2.81 and 2.90 Å, respectively.

Interestingly, compound **3d** docked into the same active pocket with the well-known MDM2 inhibitor Nutlin-2. [20]

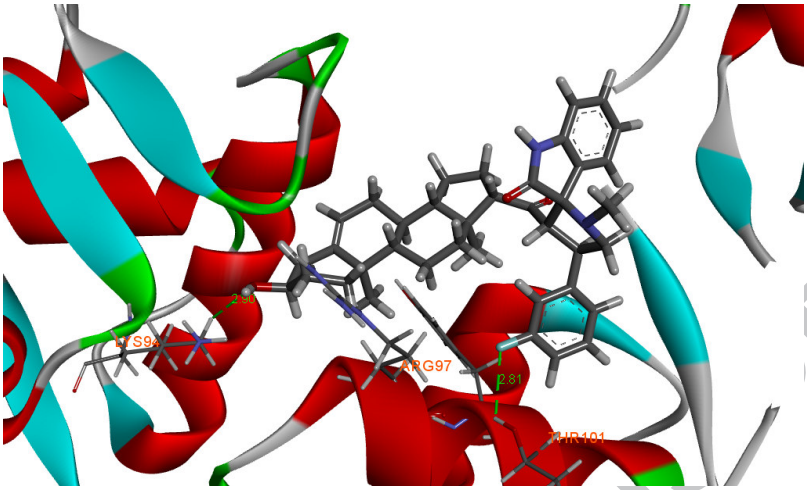


Fig. 6 Binding models of compound **3d** with MDM2 active site 1RV1.

3. Conclusions

In summary, a series of novel steroidal spirooxindoles were efficiently synthesized from commercially available pregnenolone using the 21-arylidenepregnenolone derivatives as the dipolarophiles following our previously reported method. The method involved the one-pot, three-component 1,3-dipolar cycloaddition of azomethine ylide generated *in situ* from the decarboxylative condensation of isatin and sarcosine and afforded the final products with high regio- and stereoselectivity. This protocol achieved the formation of two C-C bonds, one C-*N* bond and the creation of one new five-membered ring and three contiguous stereocenters, one of which is quaternary. Biological evaluation showed that these synthesized steroidal spirooxindoles possessed moderate to good antiproliferative activities against four human cancer cell lines and some of them were more potent than 5-FU. Among them, compounds **3e** and **3f** showed the best antiproliferative activity against MCF-7 cells (IC_{50} = 4.0 and 3.9 μM, respectively). Further investigation showed that compound **3d** caused the cellular early apoptosis and cell cycle arrest at G2/M phase in a concentration-dependent manner. Docking analysis showed that compound **3d** may interrupt the p53-MDM2 interaction by forming two hydrogen bonds in the active pocket that the previously reported MDM2 inhibitor Nutlin-2 targets.

4. Experimental section

4.1. General

Reagents were purchased and used without further purification. Thin layer chromatography (TLC) was carried out on glass plates coated with silica gel and visualized by UV light (254 nm). The final products were purified by column chromatography. Melting points were determined on an X-5 micromelting apparatus. All the NMR spectra were recorded with a Bruker DPX 400 MHz spectrometer with

TMS as the internal standard in $CDCl_3$. Chemical shifts are given as δ ppm values relative to TMS (*Most of the peaks due to the steroidal skeleton are merged and could not be differentiated. Thus, the δ values of only those peaks that distinguish the product and could easily be differentiated are reported*). High-resolution mass spectra were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionization (ESI).

*4.2. General procedure for the synthesis of 21-arylidenepregnenolone derivatives (**2a-h**)*

A mixture of pregnenolone (2.0 mmol), aromatic aldehyde (2.1 mmol) and KF/Al_2O_3 (2.0 mmol) in EtOH (20 mL) was heated under reflux for about 2 h. Upon completion of the reaction (monitored by TLC, petroleum ether/EtOAc = 2/1), the slurry was filtered and the residue was washed thoroughly with CH_2Cl_2. The filtrate was concentrated under reduced pressure, and the solid obtained was crystallized from EtOH or MeOH to give the 21-arylidenepregnenolone derivatives **2a-h**. All the intermediates are known compounds and only characterized by ^1H and ^{13}C NMR.

*4.2.1. (21E)-3β-Hydroxy-21-benzylidenepregn-5-en-20-one (**2a**)*

White solid, yield: 90 %, mp 107.1-109.2 °C. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.55 (dd, *J* = 9.7, 5.9 Hz, 2H), 6.49 (d, *J* = 16.0 Hz, 1H), 7.43-7.32 (m, 3H), 6.78 (d, *J* = 16.0 Hz, 1H), 5.36 (s, 1H), 3.61-3.44 (m, 1H), 2.86 (t, *J* = 8.7 Hz, 1H), 1.01 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 200.4, 141.5, 140.8, 134.8, 130.3, 128.9, 128.3, 126.8, 121.4, 71.7, 63.7, 62.1, 57.2, 50.0, 45.0, 44.0, 42.2, 38.8, 37.3, 36.5, 32.0, 31.9, 31.6, 24.5, 22.8, 21.1, 19.4, 13.2.

*4.2.2. (21E)-3β-Hydroxy-21-(4'-isopropylbenzylidene) pregn-5-en-20-one (**2b**)*

White solid, yield: 90 %, mp 89.9-90.8 °C. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.59-7.43 (m, 3H), 7.25 (d, 2H), 6.73 (dd, *J* = 15.8, 12.1 Hz, 1H), 5.35 (s, 1H), 3.62-3.40 (m, 1H), 2.95-2.90 (m, 1H), 1.00 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 199.7, 150.9, 140.8, 140.1, 131.7, 127.7, 126.3, 125.3, 120.7, 71.0, 61.2, 58.3, 56.5, 50.2, 49.4, 48.8, 45.4, 44.2, 41.5, 38.4, 36.5, 35.8, 34.8, 33.4, 31.3, 31.1, 30.9, 25.4, 24.0, 23.5, 22.0, 20.4, 20.3, 18.7, 12.7.

*4.2.3. (21E)-3β-Hydroxy-21-(2'-fluorobenzylidene) pregn-5-en-20-one (**2c**)*

White solid, yield: 92 %, mp 130.8-131.7°C. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.66 (d, *J* = 16.1 Hz, 1H), 7.61-7.52 (m, 1H), 7.36 (dd, *J* = 7.3, 1.5 Hz, 1H), 7.16 (t, *J* = 7.5 Hz, 1H), 7.13-7.05 (m, 1H), 6.87 (d, *J* = 16.1 Hz, 1H), 5.35 (t, *J* = 6.1 Hz, 1H), 3.59-3.44 (m, 1H), 2.88 (t, *J* = 8.8 Hz, 1H), 1.00 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.6, 161.7 (J_{C-F} = 253 Hz), 140.9, 134.2, 131.7 (J_{C-F} = 9 Hz), 129.4 (J_{C-F} = 7 Hz), 124.5, 123.0 (J_{C-F} = 12 Hz), 121.7, 121.5, 116.3 (J_{C-F} = 22 Hz), 116.2, 71.7, 62.0, 57.3, 50.1, 45.1, 42.3, 39.1, 37.3, 36.6, 32.1, 31.9, 31.7, 24.8, 22.8, 21.2, 19.5, 13.5.

*4.2.4. (21E)-3β-Hydroxy-21-(3'-fluorobenzylidene) pregn-5-en-20-one (**2d**)*

White solid, yield: 92 %，mp 154.9-155.8°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (d, *J* = 15.9 Hz, 1H), 7.35 (ddd, *J* = 18.6, 9.3, 6.7 Hz, 2H), 7.25 (dd, *J* = 9.7, 2.0 Hz, 1H), 7.13 – 7.04 (m, 1H), 6.76 (d, *J* = 15.9 Hz, 1H), 5.36 (d, *J* = 5.2 Hz, 1H), 3.61 – 3.47 (m, 1H), 2.84 (t, *J* = 8.8 Hz, 1H), 1.00 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 200.1, 163.0(J_{C-F} = 245 Hz), 140.7, 140.0, 137.1(J_{C-F} = 7 Hz), 130.4(J_{C-F} = 8 Hz), 127.8, 124.3(J_{C-F} = 3 Hz), 121.3, 117.1(J_{C-F} = 22 Hz), 114.3(J_{C-F} = 21 Hz),71.6, 62.2, 57.1, 50.0, 45.0, 42.2, 39.1, 37.2, 36.5, 32.0, 31.8, 31.6, 24.6, 22.7, 21.1, 19.3, 13.4.

*4.2.5. (21E)-3β-Hydroxy-21-(4'-chlorobenzylidene) pregn-5-en-20-one (**2e**)*

White solid, yield: 94%, mp 130.6-131.7°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.51-7.47 (m, 3H), 7.36 (d, *J* = 8 Hz, 1H), 6.73 (d, *J* = 16.0 Hz, 1H), 5.35 (t, *J* = 6.4 Hz, 1H), 3.51 (m, *J* = 1H), 2.84 (t, *J* = 8.8 Hz, 1H), 1.00 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 199.4, 140.1, 139.3, 135.4, 132.6, 128.7, 128.4, 126.4, 120.7, 71.0, 61.5, 56.5, 49.3, 44.3, 41.5, 38.4, 36.5, 35.8, 31.3, 31.1, 30.9, 23.9, 22.0, 20.4, 18.7, 12.7.

*4.2.6. (21E)-3β-Hydroxy-21-(4'-bromobenzylidene) pregn-5-en-20-one (**2f**)*

White solid, yield: 95%, mp 156.2-157.8°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.52 (d, *J* = 8.4 Hz, 2H), 7.47 (d, *J* = 15.9 Hz, 1H), 7.41 (d, *J* = 8.4 Hz, 2H), 6.76 (d, *J* = 15.9 Hz, 1H), 5.36 (d, *J* = 4.9 Hz, 1H), 3.53 (s, 1H), 2.84 (t, *J* = 8.7 Hz, 1H), 1.00 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 200.2, 140.8, 140.1, 133.7, 132.1, 129.6, 127.2, 124.5, 121.4, 71.6, 62.2, 57.1, 50.0, 45.0, 42.2, 39.1, 37.2, 36.5, 32.0, 31.8, 31.6, 24.6, 22.7, 21.1, 19.4, 13.4.

*4.2.7. (21E)-3β-Hydroxy-21-(3'-nitrobenzylidene) pregn-5-en-20-one (**2g**)*

White solid, yield: 95%, mp 168.0-169.1°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.40 (s, 1H), 8.23 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.85 (d, *J* = 7.7 Hz, 1H), 7.58 (dd, *J* = 16.0, 8.2 Hz, 2H), 6.88 (d, *J* = 16.0 Hz, 1H), 5.36 (d, *J* = 5.1 Hz, 1H), 3.62-3.42 (m, 1H), 2.87 (t, *J* = 8.7 Hz, 1H), 1.00 (s, 3H), 0.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 200.1, 149.0, 141.0, 138.7, 136.9, 134.3, 130.2, 129.4, 124.7, 122.6, 121.6, 71.9, 62.7, 57.4, 50.3, 45.4, 42.5, 39.4, 37.5, 36.8, 32.3, 32.1, 31.8, 24.9, 23.0, 21.3, 19.6, 13.8.

*4.2.8. (21E)-3β-Hydroxy-21-(1'-naphthylidene) pregn-5-en-20-one (**2h**)*

White solid, yield: 95%，mp 92.5-93.2°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.40 (d, *J* = 15.7 Hz, 1H), 8.21 (d, *J* = 8.2 Hz, 1H,), 7.88 (t, *J* = 7.7 Hz, 2H), 7.78 (d, *J* = 7.2 Hz, 1H), 7.62-7.44 (m, 3H), 6.86 (d, *J* = 15.7 Hz, 1H), 5.36 (d, *J* = 5.1 Hz, 1H), 3.58-3.43 (m, 1H), 2.90 (t, *J* = 8.7 Hz, 1H), 0.99 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 200.3, 140.8, 138.4, 133.7, 132.3, 131.8, 130.6, 129.4, 128.8, 126.9, 126.3, 125.5, 124.9, 123.5, 121.4, 71.7, 62.3, 57.2, 50.1, 45.1, 42.3, 39.3, 37.3, 36.6, 32.1, 31.9, 31.6, 24.7, 22.8, 21.2, 19.4, 13.6.

*4.3. General procedure for the synthesis of steroidal spirooxindoles (**3a-h**)*

To a mixture of 1,4-dioxane/MeOH (10 mL/10 mL) were added the 21-arylidenepregnenolone (1 mmol), isatin (1.5 mmol) and sarcosine (2.0 mmol), and then the solution was kept under reflux for about 5 h. Upon completion of the reaction (monitored by TLC, petroleum ether/EtOAc = 2/1), the solvent was removed under vacuum, the resulting residue was subjected to column chromatography to give the corresponding product.

*4.3.1. Compound **3a***

White solid, yield: 65 %, mp 162.1-162.7 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (brs, 1H), 7.48 (d, *J* = 8.0 Hz, 2H), 7.34-7.26 (m, 2H), 7.22 (dd, *J* = 7.4, 6.1 Hz, 2H), 7.09-6.98 (m, 2H), 6.89 (dd, *J* = 7.5, 2.7 Hz, 1H), 5.21 (s, 1H), 4.29 (dt, *J* = 8.5, 6.3 Hz, 1H), 3.77 (d, *J* = 9.5 Hz, 1H), 3.65-3.57 (m, 1H), 3.54-3.42 (m, 1H), 3.35 (t, *J* = 8.0 Hz, 1H), 2.19 (s, 3H), 0.87 (s, 4H), 0.07 (s, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.6, 180.7, 141.4, 140.9, 140.7, 129.3, 128.5, 128.4, 127.6, 127.3, 126.8, 123.1, 121.2, 109.7, 72.8, 71.7, 69.6, 63.8, 57.0, 49.8, 45.0, 44.2, 42.1, 38.8, 37.3, 36.4, 34.7, 31.6, 30.9, 24.2, 22.4, 19.3, 13.3. HRMS (ESI): *m/z* calcd for $C_{38}H_{47}N_2O_3$ $(M+H)^+$, 579.3587; found, 579.3581.

*4.3.2. Compound **3b***

White solid, yield: 80 %, mp 158.9-159.4 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.99 (s, 1H), 7.40 (d, *J* = 8.1 Hz, 2H), 7.20 (m, 1H), 7.15 (d, *J* = 8.1 Hz, 2H), 7.07 (d, *J* = 7.2 Hz, 1H), 7.01 (t, *J* = 7.5 Hz, 1H), 6.88 (d, *J* = 7.5 Hz, 1H), 5.22 (s, 1H), 4.27 (m, 1H), 3.72 (d, *J* = 9.5 Hz, 1H), 3.63 (t, *J* = 9.5 Hz, 1H), 3.49 (dd, *J* = 9.5, 6.1 Hz, 1H), 3.34 (dd, *J* = 8.4, 7.6 Hz, 1H), 2.87 (dt, *J* = 13.7, 6.8 Hz, 1H), 2.19 (s, 3H), 1.21 (d, *J* = 6.9 Hz, 6H), 0.87 (s, 3H), 0.08 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.2, 181.1, 147.7, 141.2, 140.9, 139.0, 129.5, 128.6, 127.8, 127.7, 126.7, 123.3, 121.5, 109.9, 73.1, 71.9, 70.2, 64.2, 60.2, 57.2, 50.0, 45.3, 44.2, 42.4, 38.8, 37.6, 36.6, 35.0, 34.0, 31.9, 31.8, 27.2, 24.4, 24.3, 22.6, 21.1, 19.5, 13.4. HRMS (ESI): *m/z* calcd for $C_{41}H_{53}N_2O_3$ $(M+H)^+$, 621.4056; found, 621.4049.

*4.3.3. Compound **3c***

White solid, yield: 70 %, mp 163.3-163.8 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.45 (s, 1H), 7.64 (t, *J* = 6.9 Hz, 1H), 7.21 (dd, *J* = 15.5, 7.8 Hz, 2H), 7.16-7.11 (m, 1H), 7.11-7.05 (m, 1H), 7.02 (m, 2H), 6.92 (d, *J* = 7.5 Hz, 2H), 5.21 (s, 1H), 4.67 (dd, *J* = 16.9, 9.5 Hz, 1H), 3.89 (d, *J* = 9.4 Hz, 1H), 3.64-3.44 (m, 2H), 3.33 (t, *J* = 7.9 Hz, 1H),2.17 (s, 3H), 0.90 (s, 3H), 0.11 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.0, 180.8, 162.2, 159.7, 141.1, 140.7, 123.0, 129.9, 129.4, 128.3, 128.1, 127.9, 127.5, 127.3, 124.3, 123.1, 121.22, 115.6, 115.4, 109.9, 72.7, 71.6, 67.8, 63.6, 57.1, 49.9, 44.9, 42.1, 39.0, 37.4, 36.7, 36.4, 34.7, 31.6, 31.5, 24.2, 22.5, 19.3, 13.4. HRMS (ESI): *m/z* calcd for $C_{38}H_{46}FN_2O_3$ $(M+H)^+$, 597.3492; found, 597.3488.

*4.3.4. Compound **3d***

White solid, yield: 74 %, mp 171.9-172.2 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.27 (brs, 1H), 7.35-7.16 (m, 4H), 7.05-6.99 (m, 2H), 6.98-6.83 (m, 2H), 5.20 (s, 1H), 4.32 (dd,

J = 16.9, 9.5 Hz, 1H), 3.73 (d, *J* = 9.3 Hz, 1H), 3.57 (t, *J* = 9.5 Hz, 1H), 3.50 (td, *J* = 10.4, 4.9 Hz, 1H), 3.35 (t, *J* = 8.0 Hz, 1H), 2.18 (s, 3H), 0.91 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.6, 180.1, 163.5, 161.0, 143.6 140.4, 140.0, 129.2, 128.7, 126.7, 126.4, 123.5, 122.4, 120.4, 114.5, 114.3, 113.1, 112.9, 109.2, 72.0, 71.0, 68.6, 63.1, 59.4, 56.4, 49.1, 44.3, 43.0, 41.4, 38.3, 36.7, 35.7, 33.9, 30.8, 23.4, 21.7, 18.5, 12.7. HRMS (ESI): *m/z* calcd for $C_{38}H_{46}FN_2O_3$ $(M+H)^+$, 597.3492; found, 597.3490.

*4.3.5. Compound **3e***

White solid, yield: 80 %, mp 165.4-165.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 (s, 1H), 7.43 (d, *J* = 8.4 Hz, 2H), 7.30-7.25 (d, , *J* = 8.4 Hz, 2H), 7.25-7.18 (m, 1H), 7.02 (m, 2H), 6.88 (d, *J* = 7.7 Hz, 1H), 5.23 (d, *J* = 4.7 Hz, 1H), 4.34-4.20 (m, 1H), 3.70 (d, *J* = 9.4 Hz, 1H), 3.50 (m, 2H), 3.32 (m, 1H), 2.17 (s, 3H), 0.90 (s, 3H), 0.11 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.4, 180.5, 140.9, 140.7, 140.0, 132.5, 129.8, 129.4, 128.6, 127.4, 127.3, 123.2, 121.2, 109.7, 72.7, 71.7, 69.3, 63.7, 60.2, 57.0, 49.8, 45.1, 45.0, 43.41, 42.10, 38.9, 37.3, 36.4, 34.7, 31.6, 31.5, 24.2, 22.5, 21.0, 19.3, 13.5. HRMS (ESI): *m/z* calcd for $C_{38}H_{46}ClN_2O_3$ $(M+H)^+$, 613.3197; found, 613.3193.

*4.3.6. Compound **3f***

White solid, yield: 80 %, mp 170.4-171.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (s, 1H), 7.43 (d, *J* = 8.5 Hz, 2H), 7.37 (d, *J* = 8.5 Hz, 2H), 7.26-7.12 (m, 1H), 7.02 (q, *J* = 7.3 Hz, 2H), 6.88 (d, *J* = 7.7 Hz, 1H), 5.23 (d, *J* = 4.6 Hz, 1H), 4.27 (dd, *J* = 16.8, 9.6 Hz, 1H), 3.70 (d, *J* = 9.2 Hz, 1H), 3.50 (m, 2H), 3.32 (t, *J* = 8.0 Hz, 1H), 2.17 (s, 3H), 0.90 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.9, 180.9, 141.4, 141.2, 141.1, 132.1, 130.7, 129.9, 127.9, 127.8, 123.7, 121.7, 121.1, 110.2, 73.2, 72.2, 69.8, 64.3, 61.0, 57.6, 50.3, 45.6, 44.0, 42.6, 39.4, 37.8, 36.9, 35.2, 32.1, 32.0, 24.7, 23.0, 21.6, 21.5, 19.8, 14.0. HRMS (ESI): *m/z* calcd for $C_{38}H_{46}BrN_2O_3$ $(M+H)^+$, 657.2692; found, 657.2686.

*4.3.7. Compound **3g***

White solid, yield: 82 %, mp 170.8-171.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.39 (t, *J* = 1.8 Hz, 1H), 8.15-8.08 (m, 1H), 7.89 (d, *J* = 7.8 Hz, 1H), 7.52 (t, *J* = 7.9 Hz, 1H), 7.29-7.22 (m, 1H), 7.10-7.00 (m, 2H), 6.94 (d, *J* = 7.7 Hz, 1H), 5.23 (d, *J* = 3.9 Hz, 1H), 4.46 (td, *J* = 9.6, 7.3 Hz, 1H), 3.76 (d, *J* = 9.3 Hz, 1H), 3.61 (t, *J* = 9.4 Hz, 1H), 3.56-3.44 (m, 1H), 3.41 (dd, *J* = 8.7, 7.3 Hz, 1H), 2.72 (s, 1H), 2.21 (s, 3H), 0.89 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.1, 180.3, 147.7, 143.1, 140.2, 140.0, 134.1, 128.9, 128.7, 126.4, 122.5, 122.4, 121.3, 120.4, 109.2, 72.6, 71.8, 71.0, 68.3, 63.0, 59.2, 56.3, 49.1, 44.4, 42.8, 41.4, 38.4, 35.6, 33.9, 30.8, 30.7, 23.4, 21.8, 20.3, 18.5, 12.9. HRMS (ESI): *m/z* calcd for $C_{38}H_{46}N_3O_5$ $(M+H)^+$, 624.3437; found, 624.3441.

*4.3.8. Compound **3h***

White solid, yield: 81 %, mp 182.3-183.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.77 (d, *J* = 8.6 Hz, 1H), 8.06 (d, *J* = 7.1 Hz, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.90

(d, J = 8.1 Hz, 1H), 7.77-7.70 (m, 1H), 7.64 (t, J = 7.7 Hz, 2H), 7.45-7.36 (m, 1H), 7.33 (d, J = 7.3 Hz, 1H), 7.20 (dd, J = 16.0, 8.9 Hz, 1H), 7.08 (d, J = 7.7 Hz, 1H), 5.42 (dd, J = 16.7, 9.1 Hz, 1H), 5.34 (d, J = 3.6 Hz, 1H), 4.24 (d, J = 9.1 Hz, 1H), 3.86 (t, J = 9.2 Hz, 1H), 3.64 (dd, J = 17.2, 8.6 Hz, 2H), 2.39 (s, 3H), 0.98 (s, 3H), 0.24 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.1, 179.7, 140.0, 139.7, 137.2, 132.9, 131.2, 128.4, 127.6, 126.6, 126.4, 126.2, 125.1, 124.6, 124.5, 124.4, 123.1, 122.2, 120.2, 108.8, 72.1, 70.8, 68.5, 62.9, 60.5, 56.2, 48.9, 44.0, 41.2, 37.9, 37.6, 36.4, 35.4, 35.3, 33.9, 31.2, 30.6, 23.2, 21.6, 20.0, 18.3, 12.1. HRMS (ESI): *m/z* calcd for $C_{42}H_{49}N_2O_3$ $(M+H)^+$, 629.3743; found, 629.3742.

4.4. Cell culturing

Human cancer cell lines were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cancer cells were maintained in RPMI1640 medium. All cell lines were purchased from the China Center for Type Culture Collection (CCTCC, China). For pharmacological investigations, 10 mM stock solutions of the tested compounds were prepared with DMSO. The highest DMSO concentration of the medium (0.1%) does not have any substantial effect on the determined cellular functions.

4.5. Antiproliferative assay

Exponentially growing cells were seeded into 96-well plates at a concentration of 5 × 10^3 cells per well. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium containing tested compounds at different concentrations. The cells were incubated for another 72 h. Then, 20 μL of MTT solution (5 mg/mL) was added to all wells and incubated for 4 h at 37 °C. The suspension was discarded and 150 μL of DMSO was added to each well. The plate was shaken to dissolve the dark blue crystal (formazan); the absorbance was measured using a microplate reader at the wavelength of 490 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves according to the inhibition ratio for each concentration [21].

4.6. Analysis of cellular apoptosis

MCF-7 cells were plated in 6-well plate (5.0 × 10^6 cells/mL) and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated for 24 h with complete medium (blank) or with compound **3d**. Cells were then harvested and the Annexin-V-FITC/PI apoptosis kit (Biovision) was used according to the manufacturer's instructions to detect apoptotic cells. Ten thousand events were collected for each sample and analyzed by Accuri C6 flow cytometer.

4.7. Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 5.0×10^6 MCF-7 cells in exponential growth were treated with different concentrations of compound **3d** for 12 h. After an incubation period, the cells were collected, centrifuged and fixed with ice-cold EtOH (70%). The cells were then treated with buffer containing RNAse A and 0.1% Triton X-100 and then stained with PI. Samples were analyzed on Accuri C6 flow cytometer (Becton, Dickinson). Data obtained from the flow cytometer was analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

4.7. Molecular modeling

The structure of the MDM2 binding site was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb). Docking simulations were carried out in two stages using AutoDock Vina designed and implemented by Dr. Oleg Trott in the Molecular Graphics Lab at The Scripps Research Institute. To run autodock, we used a searching grid extended over the selected target proteins; polar hydrogens were added to the ligand moieties. Kollman charges were assigned and atomic solvation parameters were added. Polar hydrogen charges of the Gasteiger-type were assigned and the nonpolar hydrogens were merged with the carbons and the internal degrees of freedom and torsions were set.

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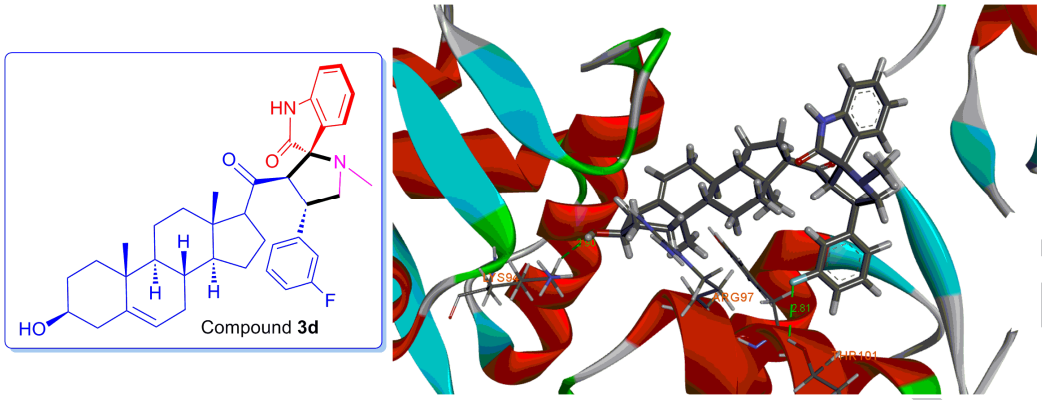

Highlights

- Spirooxindoles are privileged scaffolds in drug discovery
- A series of novel steroidal spirooxindoles were synthesized
- Most of these compounds exhibited moderate to good antiproliferative activity
- Some of them were more potent than 5-FU
- Compound **3d** caused cell cycle arrest at G2/M phase and early apoptosis