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Synthesis and biological evaluation of spiro[cyclopropane-1,3′-indolin]-2′-ones as potential anticancer agents

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ABSTRACT

Libraries of spiro[cyclopropane-1,3′-indolin]-2′-ones were synthesized and evaluated for their biological activity against five different human cancer cell lines HT-29 (colon cancer), DU-145 (prostate cancer), Hela (cervical cancer), A-549 (Lung cancer), and MCF-7 (breast cancer). Many compounds of the series exhibited promising anticancer activity (IC_{50} <20 μM) against the studied cell lines. Based on the screening results, a structure activity relationship (SAR) of the pharmacophore was proposed. Among the series compound **6b** and **6u** showed significant activity against human prostate cancer cell line, DU-145. Flow cytometric analysis showed that these two compounds arrested the cell cycle in the G0/G1 phase leading to caspase-3 dependent apoptotic cell death. Further, measurement of mitochondrial membrane potential and Annexin V-FITC assay also suggested that **6b** and **6u** induced cell death by apoptosis.

Cancer is one of the most serious clinical problems in the world with its increasing incidence every year. Despite avoiding risk factors such as tobacco, overweight and obesity, and preventive managements such as dietary, medication and vaccination, the disease still affects millions of patients worldwide. Apart from surgery and radiation treatments, chemotherapy has been proven very useful in cancer therapy.[1] Most of the anticancer drugs generally act on metabolically active or rapidly proliferating cells, and suffer from poor selectivity between cancer and normal cells. The high toxicity and poor tolerance of the current anticancer drugs have led to the quest for novel agents with high efficiency, low toxicity, and minimum undesirable side effects. Therefore, new molecular libraries are currently being investigated as newer anticancer agents.[2]

Spirooxindole represents an important class of heterocyclic motif with promising biological and pharmacological relevance. In recent years, numerous spirooxindoles were reported as promising anticancer agents (Fig. 1). For instance, spiro(oxindole-3,3′-thiazolidine)-derivative **I** has been found as potential anticancer agents acting through modulation of p53 activity.[3] Spiro[indole-pyridothiazine] **II** was reported as potent antiproliferative agents.[4] Recently, spirooxindole-pyrrolidine **III**[5] and isoxazolidine spirocyclic oxindole **IV**[6] were found as promising anticancer agents. Moreover 4-thiazolidinone-, pyrazoline-, and isatin-based conjugates type **V** have also been found as potential antitumor agents.[7] Recently we investigated the reactions of diazo-compounds with electron deficient alkenes to yield pyrazoles and cyclopropanes.[8] In order to fully explore the scope of a new synthetic strategy, understanding its mechanistic aspect is very important. The present study describes synthesis of spiro[cyclopropane-1,3′-indolin]-2′-one derivatives **VI** using catalyst-free EDA strategy, their anticancer activity and the mechanistic aspects of the reaction.

The overall strategy for the synthesis of spiro[cyclopropane-1,3′-indolin]-2′-ones is shown in Scheme 1. Substituted isatin **1** was converted to (*E*)-ethyl 2-(2-oxoindolin-3-ylidene)acetate **3** by treating it with (ethoxycarbonylmethylene)-triphenylphosphorane. 3-Methyleneindolin-2-one derivatives **5** were synthesized from isatin **1** and acetophenone **2** in a two step procedure depicted in Scheme 1. Next **3**/**5** were refluxed with EDA in THF for 24 h to yield diastereomerically pure spiro[cyclopropane-1,3′-indolin]-2′-ones **4**/**6**. The carbonyl groups of **6** were reduced to alcohol **7** by treating them with sodium borohydride in ethanol. All the synthesized compounds were characterized by Mass/HRMS, IR, ^{1}H and ^{13}C NMR spectroscopy.

Stereoselective catalyst-free cyclopropanation of electron deficient alkenes using EDA were proposed to follow an ionic mechanism involving Michael initiated ring closure (MIRC).[8a,b]

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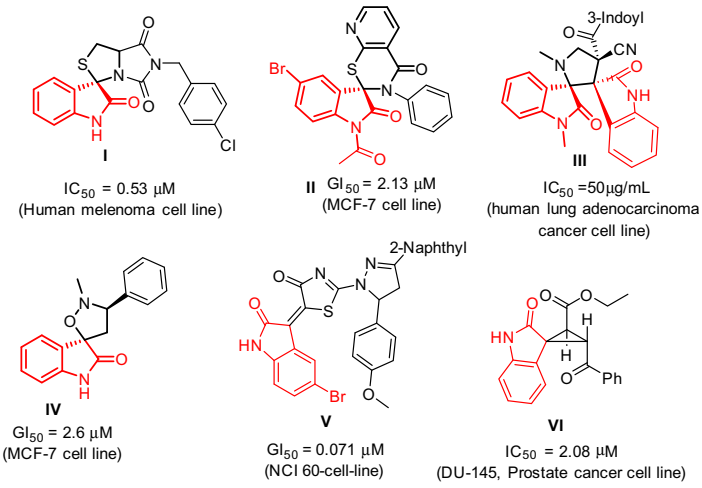
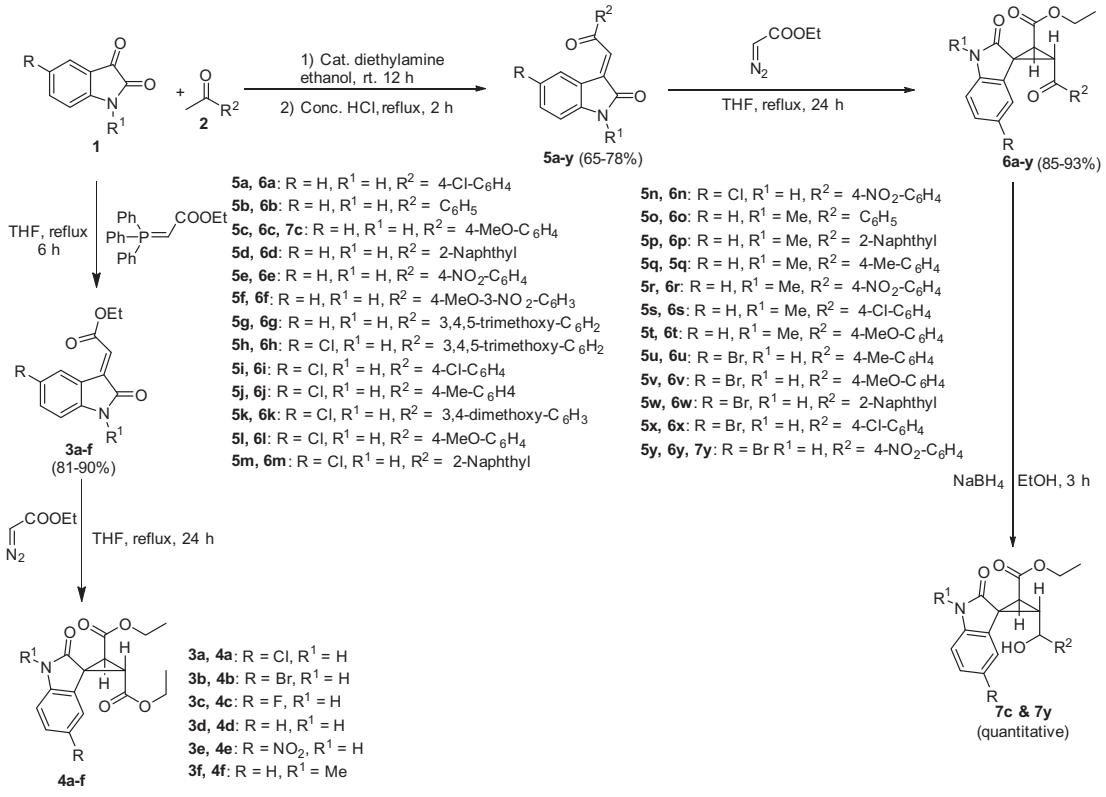
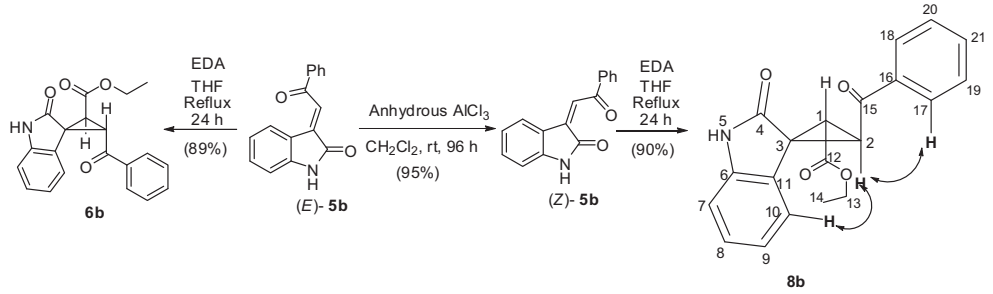


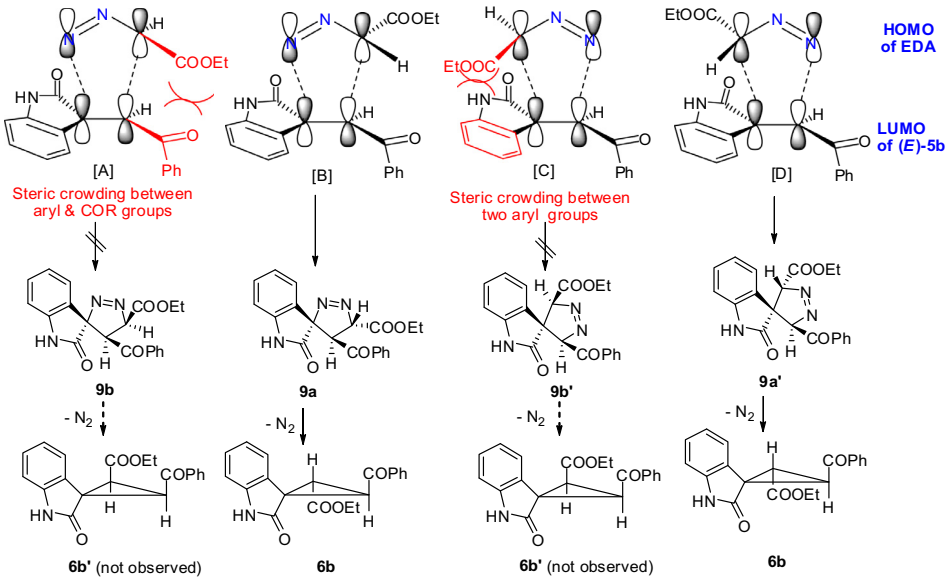
Figure 1. Chemical structures and anticancer activities of spiroindolines **I–IV**, oxindole conjugates **V** and spiro[cyclopropane-1,3′-indolin]-2′-ones **VI**.



Scheme 1. Schematic illustration for the synthesis of spiro[cyclopropane-1,3′-indolin]-2′-one derivatives.



Scheme 2. Conversion of (*E*)-**5b** to (*Z*)-**5b**, their reactions with EDA and characteristic Nuclear Overhauser Effects (NOE's) of compound **8b**.



Scheme 3. Proposed mechanism for the catalyst-free diastereoselective cyclopropanation of (*E*)-**5b** based on HOMO-LUMO controlled 1,3-polar cycloaddition/ring contraction sequence.

Table 1
Cytotoxic effects of compounds **6a–y**, **4a–f**, **7c**, **7y** and **8b** on various cancer cell lines

Entry	Compound	IC_{50} (μM) [a]					
		HT-29[b]	DU-145[c]	Hela[d]	A-549[e]	MCF-7[f]	HEK-293[g]
1	**6a**	23.72	15.84	19.95	12.88	18.62	NT
2	**6b**	**3.89**	**2.08**	**7.50**	**5.12**	**3.98**	**64.56**
3	**6c**	14.69	17.37	26.30	9.77	19.09	NT
4	**6d**	17.96	17.17	20.04	12.58	31.06	NT
5	**6e**	25.11	19.84	41.31	19.05	22.07	NT
6	**6f**	7.24	6.36	7.76	4.791	9.77	NT
7	**6g**	8.51	15.84	32.82	17.7	50.11	NT
8	**6h**	25.06	21.90	39.81	24.14	37.54	NT
9	**6i**	79.43	>100	>100	32.44	30.82	NT
10	**6j**	>100	>100	>100	>100	90.2	NT
11	**6k**	9.92	8.31	12.97	7.07	20.31	NT
12	**6l**	9.54	5.01	9.12	6.421	9.33	NT
13	**6m**	**5.01**	**5.24**	**7.76**	**6.16**	**4.89**	NT
14	**6n**	**2.95**	**2.88**	**10.65**	**3.59**	**4.26**	NT
15	**6o**	16.10	7.94	19.49	7.24	13.18	NT
16	**6p**	>100	>100	>100	98.47	75.85	NT
17	**6q**	23.95	7.24	33.47	5.49	24.70	NT
18	**6r**	19.74	18.93	51.22	26.08	37.15	NT
19	**6s**	8.51	6.30	12.58	6.556	12.58	NT
20	**6t**	13.19	15.03	15.36	5.88	18.19	NT
21	**6u**	**1.87**	**1.86**	**16.09**	**4.56**	**3.63**	**79.43**
22	**6v**	33.71	31.65	45.08	79.40	41.61	NT
23	**6w**	79.43	36.86	>100	25.00	29.28	NT
24	**6x**	8.47	8.12	62.13	15.84	12.24	NT
25	**6y**	42.65	31.62	68.88	30.50	>100	NT
26	**4a**	22.88	19.89	25.1	29.0	24.04	NT
27	**4b**	30.90	50.73	62.8	>100	>100	NT
28	**4c**	36.60	24.32	84.2	78.2	36.75	NT
29	**4d**	18.94	18.61	61.3	>100	57.54	NT
30	**4e**	50.11	38.01	32.9	>100	71.58	NT
31	**4f**	26.91	39.80	32.2	74.2	32.13	NT
32	**7c**	**2.29**	**1.94**	**5.78**	**27.54**	**4.78**	NT
33	**7y**	23.44	27.77	28.84	21.10	>100	NT
34	**8b**	**2.75**	**1.69**	**7.24**	**4.16**	**2.95**	NT
35	Doxorubicin	**0.93**	**1.94**	**1.51**	**1.58**	**0.63**	NT

The bold value signifies the potent cytotoxic effect against the corresponding cell lines.
NT = Not tested.
[a] 50% Inhibitory concentration after 48 h of drug treatment and the values are average of three individual experiments.
[b] Colon cancer.
[c] Prostate cancer.
[d] Cervical cancer.
[e] Lung cancer.
[f] Breast cancer.
[g] Normal cell line.

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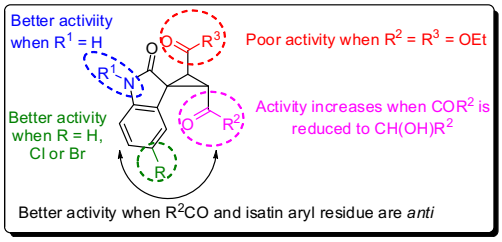
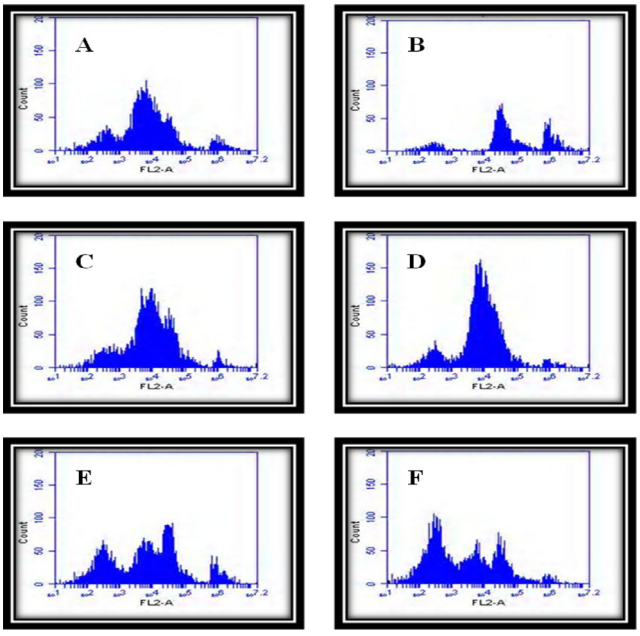


Figure 2. SAR for spiro[cycloptopane-1,3′-indolin]-2′-ones.

However, further experimental results (depicted in Scheme 2) put the MIRC mechanism in doubt and suggested a concerted 1,3-dipolar cycloaddition/ring contraction sequence with retention of configuration for the same. All the synthesized 3-methyleneindolin-2-ones **5** had *E*-configuration across the C-C double bond. For our study (*E*)-**5b** was taken as a model substrate as it easily gets converted to (*Z*)-**5b** upon treatment with anhydrous $AlCl_3$.[9] Next (*Z*)-**5b** was refluxed with EDA in THF for 24 h leading to the formation of a new product **8b** (Scheme 2). The structure of **8b** was confirmed by its characteristic NOEs. Briefly, the presence of NOE cross peak between H_{17}–H_2 and H_2–H_{10} indicated *trans*-orientation of the phenyl residue of isatin and benzoyl group. Furthermore, the presence of NOE cross peak H_2-H_{10} along with coupling constant (*J*) of 8.3 Hz for H_1-H_2 indicated *trans*-orientation of the cyclopropane ring hydrogen atoms. The formation of **6b** from (*E*)-**5b** and **8b** from (*Z*)-**5b** clearly indicated that the stereochemistry of (*E*)-**5b** and (*Z*)-**5b** were retained in the final product. In the lights of these experiments, formation of **6b** and **8b** can be explained by a plausible mechanism based on a 1,3-dipolar cycloaddition/ring contraction sequence with retention of configuration as depicted in Scheme 3. Here we assume that the reaction proceeds through interaction of highest occupied molecular orbital (HOMO) of EDA and lowest unoccupied molecular orbital (LUMO) of the alkene. As depicted in Scheme 3, path A and C are not favoured due to steric crowding between ethoxycarbonyl and aroyl group (path A), and ethoxycarbonyl and indoline residue (path C), and agree well with the non-observation of the *cis*-product **6b′**. In the same way, the proposed mechanism depicted in Scheme 3 can be used to explain the observed stereoselectivity of the compounds **4a–f**, **6a–y** and **8b**.

All the compounds (**6a–y**, **4a–f**, **7c**, **7y** and **8b**) were tested for their anticancer activity in a panel of five human cancer cell lines HT-29 (colon cancer), DU-145 (prostate cancer), Hela (cervical cancer), A-549 (Lung cancer) and MCF-7 (breast cancer). In order to screen the anticancer activity, MTT assay[10] was used for accessing cell viability. The values obtained were compared to the standard drug doxorubicin. The screening results are shown in Table 1 and expressed as IC_{50} values. The screening results suggested that the compounds **6b**, **6m**, **6n**, **6u**, **7c**, and **8b** showed potential cytotoxic activity (IC_{50} <5 μM) against the studied cell lines. Compounds of series **6** bearing H, Cl and Br atoms at 5-position of isatin residue (R group) showed superior anticancer activity. It was also observed that compounds having free-NH group exhibited better cytotoxic activity than those with N-Me group. The spiro[cyclopropane-1,3′-indolin]-2′-ones with bis-ethoxycarbonyl group on cyclopropane ring **4a–f** did not show good anticancer activity. The compounds **7c** and **7y** showed better anticancer activity than their corresponding precursors (**6c** and **6y**). It is plausibly due to increased bioavailability of alcohols compared to those with corresponding ketones. Compound **8b** exhibited superior anticancer



Sample	Sub G1 %	G0/G1 %	S %	G2/M %
A: Control (DU-145)	9.12	79.81	1.74	6.51
B:Nocodazole (1.5 μM)	9.25	57.63	5.61	26.59
C: **6b** (1.5 μM)	7.77	84.93	2.11	4.03
D: **6b** (3 μM)	16.58	70.57	2.27	8.93
E: **6u** (1.5 μM)	8.80	85.57	1.35	3.40
F: **6u** (3 μM)	26.17	64.01	2.10	3.16

Figure 3. Cell cycle analysis of **6b** and **6u** on DU-145 cells. (A) Control cells (DU-145), (B) Nocodazole (1.5 μM), (C) **6b** (1.5 μM), (D) **6b** (3 μM), (E) **6u** (1.5 μM) and (F) **6u** (3 μM).

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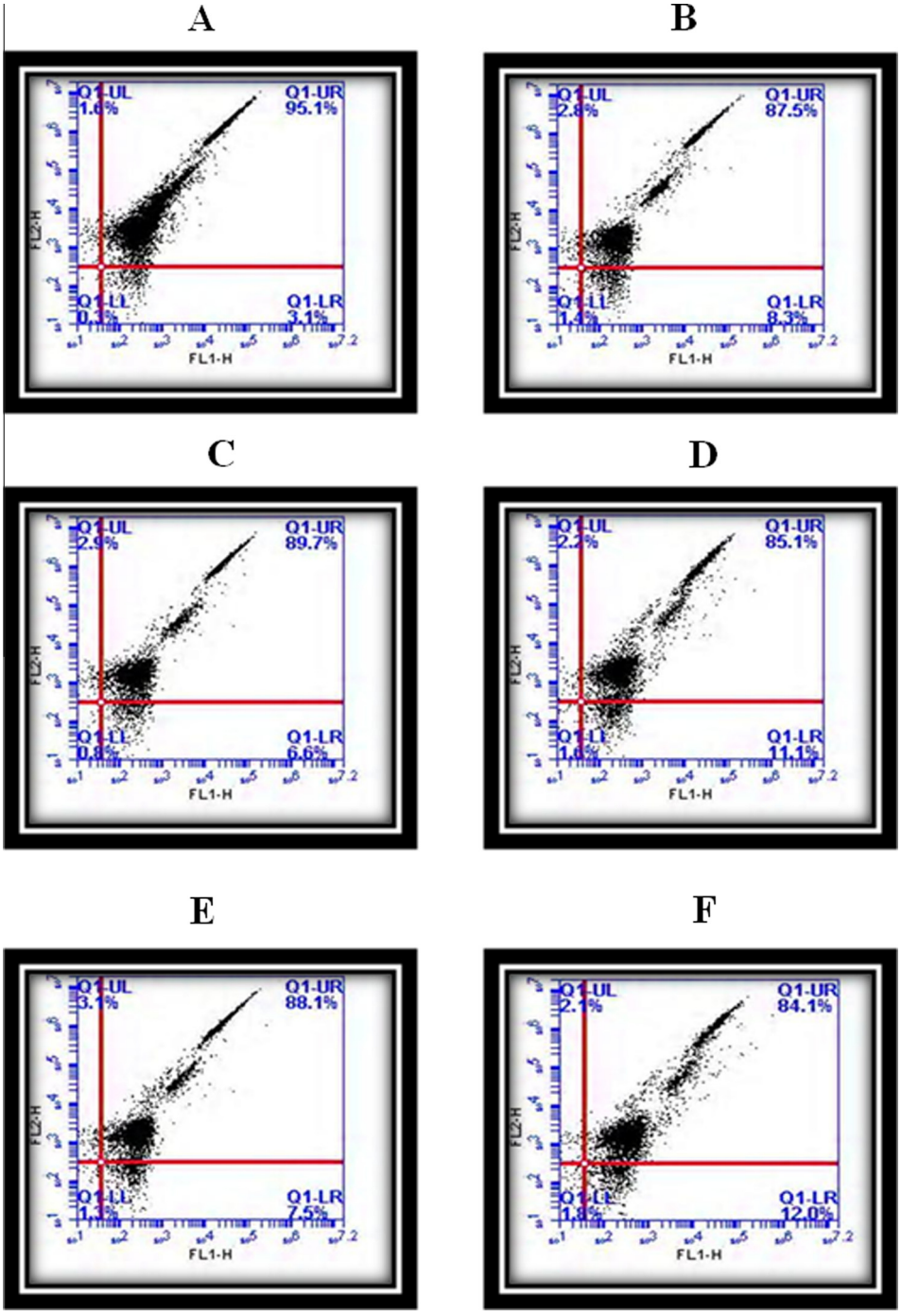

Figure 4. Drops in membrane potential ($\Delta\Psi m$) were assessed by JC-1 staining of DU-145 cells treated with compounds **6b** and **6u** and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson). (A) Control cells (DU-145), (B) Nocodazole (1.5 μM), (C) **6b** (1.5 μM), (D) **6b** (3 μM), (E) **6u** (1.5 μM) and (F) **6u** (3 μM).

activity to its isomer **6b**. Based on these observations, a SAR of spiro[cyclopropane-1,3′-indolin]-2′-ones was proposed as depicted in Figure 2.

In order to study the possible mechanism of action for these compounds, **6b** and **6u** were chosen as model substrates and DU-145 as a model cell line for subsequent experiments.

Cell cycle analysis: Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of cell cycle or by induction of apoptosis or a combined effect of both cycle block and apoptosis.[11,12] In vitro screening results revealed that compounds **6b** and **6u** showed significant activity against human prostate cancer cell line, DU-145. Therefore, it was considered of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. In this study DU-145 cells were treated with these compounds at concentrations of 1.5 and 3 μM for 48 h. The data obtained clearly indicated that this compounds arrested the cell cycle at G0/G1 phase at 1.5 μM. Interestingly, when the concentration was increased from 1.5 μM to 3 μM it was observed that the percentage of cells in G0/G1 phase was decreased and accumulation of cells in subG1 phase increased, which indicates the onset of apoptosis[13] (Fig. 3).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$): The maintenance of mitochondrial membrane potential (($\Delta\Psi m$) is significant for mitochondrial integrity and bio-energetic function.[14]

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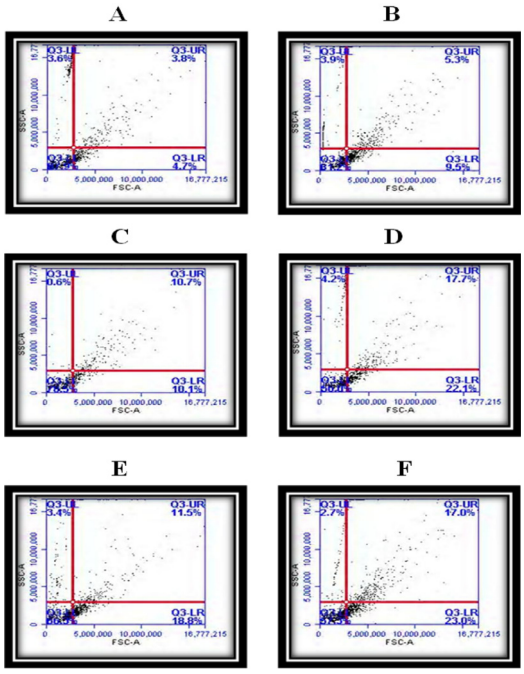

Sample	UL %	UR %	LL%	LR %
A: Control	3.56	3.83	87.88	4.73
B: Nocodazole (1.5 μM)	3.92	5.35	81.21	9.52
C: **6b** (1.5 μM)	0.62	10.74	78.52	10.12
D: **6b** (3 μM)	4.19	17.66	56.01	22.15
E: **6u** (1.5 μM)	3.41	11.50	66.31	18.78
F: **6u** (3 μM)	2.74	17.00	57.28	22.98

Figure 5. Annexin V-FITC staining. (A) Control cells (DU-145), (B) Nocodazole (1.5 μM), (C) **6b** (1.5 μM), (D) **6b** (3 μM), (E) **6u** (1.5 μM) and (F) **6u** (3 μM).

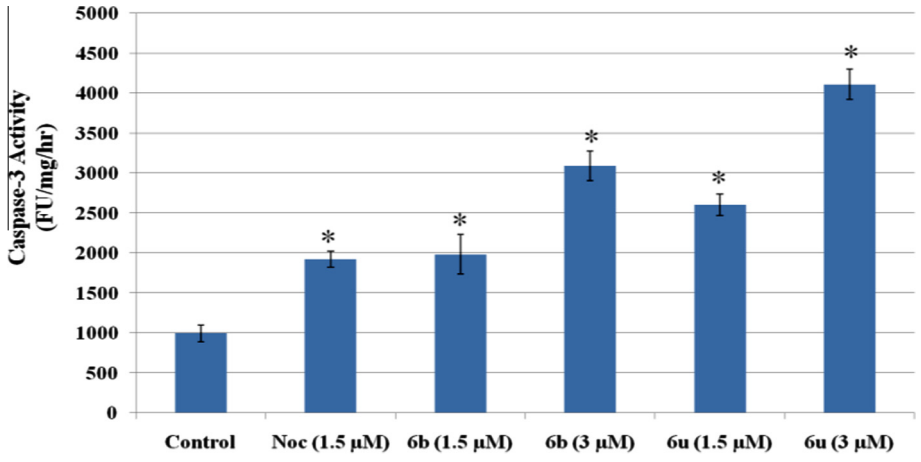

Figure 6. Effect of compounds **6b** and **6u** on caspase-3 activity: values indicated are the mean ± SD of two different experiments performed in triplicates; $^{*}P$ <0.005 compared to control.

Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm), are key events that take place during drug-induced apoptosis. Mitochondrial injury by **6b** and **6u** was evaluated by detecting drops in mitochondrial membrane potential (ΔΨm). In this study we have investigated the involvement of mitochondria in the induction of apoptosis by **6b** and **6u**. After 48 h of drug treatment with these compounds at 1.5 and 3 μM concentrations, it was observed that reduced mitochondrial membrane potential (ΔΨm) of DU-145 cells, assessed by JC-1 staining (Fig. 4).

Annexin V-FITC for apoptosis: The apoptotic effect of **6b** and **6u** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[15] to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis. In this study DU-145 cells were treated with compounds **6b** and **6u** for 48 h at 1.5 and 3 μM concentrations to examine the apoptotic effect. It was observed that these compounds showed significant apoptosis against DU-145 cells as shown in Figure 5.

Caspase-3 activity: Activation of caspases plays a vital role for the initiation and execution of the apoptotic process.[16] Among the caspases, caspase-3 is one of the key effector caspase that cleave multiple proteins in cells and lead to apoptotic cell

death.[17,18] In this context, DU-145 cells were treated with compounds **6b** and **6u** at 1.5 and 3 μM concentration for 48 h. Results indicate that there was nearly 2 to 3-fold induction in caspase-3 levels compared to the control (Fig. 6).

In summary, we report the synthesis and anticancer activity of a series of spiro[cyclopropane-1,3′-indolin]-2′-ones against five human cancer cell lines, namely HT-29 (colon), DU-145 (prostate), Hela (cervical), A549 (lung) and MCF-7 (breast). The experimental results suggested the 1,3-dipolar cycloaddition/ring contraction mechanism for the diastereoselective cyclopropanation of 3-methyleneindolin-2-ones. Compound **6b** and **6u** showed significant anticancer activity against human prostate cancer cell line, DU-145. Detailed biological studies like, cell cycle analysis showed that these compounds arrest the cell cycle at G0/G1 phase and induced cell death by apoptosis. It was further confirmed by mitochondrial membrane potential, Annexin V-FITC analysis and Caspase-3 activity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.056.

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General experimental procedure for the synthesis of compounds **5a–y**, **3a–f** and **8b**: To a 25 ml round bottom flask, 3-methyleneindolin-2-ones (1 mmol), ethyl diazoacetate (1.1 mmol), and tetrahydrofuran (10 ml) were taken and the reaction mixture was refluxed overnight. After completion, the reaction mixture was evaporated to yield crude product which was further purified by column chromatography using ethyl acetate-hexane in increasing polarity to yield desired compounds. Characterization data for ethyl-2-benzoyl-2′-oxospiro[cyclopropane-1,3′-indoline]-3-carboxylate **8b**: Physical appearance: white solid; mp 169–170 °C; R_f = 0.30 (50% EtOAc/*n*-Hexane); IR (KBr, cm^{-1}): 3219, 3059, 2978, 2925, 1734, 1708, 1688, 1617, 1471, 1447, 1362, 1342, 1310, 1201, 1165; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.84 (d, *J* = 7.8 Hz, 2H), 7.56–7.49 (m, 2H), 7.39–7.31 (m, 3H), 7.12 (t, *J* = 7.3 Hz, 1H), 6.95 (d, *J* = 7.8 Hz, 1H), 4.36–4.08 (m, 2H), 3.84 (d, *J* = 7.9 Hz, 1H), 3.49 (d, *J* = 7.8 Hz, 1H), 1.27 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO) δ 189.56, 171.90, 166.84, 142.21, 135.25, 132.83, 128.01, 127.88, 127.49, 123.61, 121.88, 121.22, 109.60, 60.87, 38.84, 38.78, 33.93, 13.45. HRMS calcd for $C_{20}H_{18}NO_4$; 336.1236, found 336.1246. General experimental procedure for the reduction of carbonyl compounds: To a 25 mL round bottom flask, carbonyl compound (1 mmol) and ethanol (10 mL) was taken under nitrogen atmosphere. Next $NaBH_4$ (5 mmol) was added to the reaction mixture in portions with stirring. After the reaction was complete, the reaction mixture was concentrated and extracted with EtOAc/saturated aq. $NaHCO_3$. The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated to yield pure products. Characterization data for ethyl 3-(hydroxy(4-methoxyphenyl)methyl)-2′-oxospiro[cyclopropane-1,3′-indoline]-2-carboxylate **7c**: Physical appearance: white solid; mp 202–204 °C; R_f = 0.38 (50% EtOAc/*n*-Hexane); IR (KBr, cm^{-1}) : 3469, 3078, 2950, 2873, 1739, 1696, 1251; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.90 (s, 1H), 7.50 (d, *J* = 8.6 Hz, 2H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.14 (d, *J* = 7.4 Hz, 1H), 7.02 (t, *J* = 7.5 Hz, 1H), 6.91 (dd, *J* = 12.8, 8.3 Hz, 3H), 4.82 (d, *J* = 9.7 Hz, 1H), 4.11 (q, *J* = 7.1 Hz, 2H), 3.82 (s, 3H), 3.14–2.95 (m, 1H), 2.90 (s, 1H), 2.72 (d, *J* = 8.1 Hz, 1H), 1.18 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$+DMSO) δ 174.22, 166.16, 158.34, 141.85, 134.84, 127.02, 126.79, 126.64, 126.21, 120.77, 113.10, 109.51, 69.11, 60.33, 54.55, 40.50, 36.75, 35.61, 13.43. HRMS calcd. for $C_{21}H_{22}NO_5$; 368.1498, found 368.1503. Characterization data for ethyl 5′-bromo-3-(hydroxy(4-nitrophenyl)methyl)-2′-oxospiro [cyclopropane-1,3′-indoline]-2-carboxylate **7y**: Physical appearance: orange solid; mp 207–208 °C; R_f = 0.48 (50% EtOAc/*n*-Hexane); IR (KBr, cm^{-1}) : 3412, 3005, 1731, 1682, 1620, 1526, 1320, 1218; ^{1}H NMR (300 MHz, $CDCl_3$+DMSO) δ 9.91 (s, 1H), 8.09 (d, *J* = 8.8 Hz, 2H), 7.59 (d, *J* = 8.7 Hz, 2H), 7.34 (d, *J* = 1.7 Hz, 1H), 7.23 (dd, *J* = 8.3, 1.9 Hz, 1H), 6.74 (d, *J* = 8.3 Hz, 1H), 5.23 (d, *J* = 4.3 Hz, 1H), 4.91 (dd, *J* = 8.0, 4.8 Hz, 1H), 4.04 (q, *J* = 7.1 Hz, 2H), 2.79 (dt, *J* = 16.3, 8.2 Hz, 1H), 1.11 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$+DMSO) δ 174.11, 166.38, 150.56, 141.57, 130.33, 128.71, 126.96, 126.68, 125.00, 123.53, 122.07, 113.91, 111.53, 69.34, 61.35, 41.12, 36.99, 36.36, 29.59, 14.05. HRMS calcd. for $C_{20}H_{18}BrN_2O_6$; 461.0348, found 461.0363.