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Scaffold hybridization in generation of indenoindolones as anticancer agents that induce apoptosis with cell cycle arrest at G2/M phase

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ABSTRACT

Scaffold hybridization of several natural and synthetic anticancer leads led to the consideration of indenoindolones as potential novel anticancer agents. A series of these compounds were prepared by a diversity-feasible synthetic method. They were found to possess anticancer activities with higher potency compared to etoposide and 5-fluorouracil in kidney cancer cells (HEK 293) and low toxicity to corresponding normal cells (Vero). They exerted apoptotic effect with blocking of cell cycle at G2/M phase.

Cancer is the second leading cause of mortality worldwide after cardiovascular diseases.[1] Chemotherapeutic treatment, although major choice, suffers from increasing resistance responses[2] among patients to existing drugs, lack of wider activities and selectivity. To overcome these, the development of novel anticancer agents is of immense demand. Towards generation of new chemical entities (NCEs), the structural simplification/modification of bioactive natural product's complex molecular architecture[3] and the generation of analogue/mimicking scaffold and hybrid[4] of chemotypes of known drugs or therapeutic agents have gained importance. In the context of generation of motif-hybrid as lead, an excellent example is azatoxin derived from topoisomerase II-targeted anticancer drugs etoposide and ellipticine.[5] Azatoxin with relevant substitution was found to possess higher inhibitory activities of topoisomerase II than etoposide or ellipticine. Recently, reported examples of scaffold-hybrids, which have been identified as promising anticancer leads, include indole-barbituric acid[6] and stilbene-coumarin.[7] As a part of our research on anticancer drug discovery, we focused at scaffold hybridization design approach. Indolo[2,3-*a*] carbazole (e.g., natural occurring rebeccamycin, arcyriaflavins A–D, staurosporine), neo-tanshinlactone (a component of Chinese traditional medicine Tanshen), indenoisoquinolines, and 3-arylindoles are known to possess anticancer activities (Fig. 1).[8] Based on these chemotypes, we considered indenoindolone as important hybrid scaffold for potential anticancer activity. Compound comprising of indenoindolone-oxime template and terminal alkyne moiety was reported to possess cytotoxicity.[9] It is noteworthy that indenoindole derivatives are known to possess various pharmaceutical activities such as inhibitors of protein kinase CK2 and antioxidant activity.[10]

In this communication, we present indenoindolones as anticancer agents that possess activities of higher potency and lower toxicity than clinically used drugs etoposide and 5-fluorouracil (5-FU) in kidney cancer cells (HEK 293). Most of the conventional anticancer drugs/agents often arrest the cell cycle in a specific phase depending on their mechanism of action. Recently, we developed N-fused imidazole as novel anticancer agents that induced apoptosis in G1/S phase.[11] The present studies of indenoindolones with experiments of nuclear staining using 4′,6-diamidino-2-phenylindole (DAPI), expression of apoptotic markers and fluorescence activated cell sorter (FACS) analysis indicated their apoptotic effect with cell cycle arrest at G2/M phase.

The organic synthesis plays important role in drug discovery process. Particularly, the convenient new synthetic methods and technologies especially towards rapid generation of molecular diversity available to synthetic and medicinal chemists help the lead generation.[12] Recently, we developed an efficient route of Friedel–Crafts 3-(2-bromo)benzoylation of indoles and intramolecular direct arylation towards the synthesis of indenoindolones.[13]

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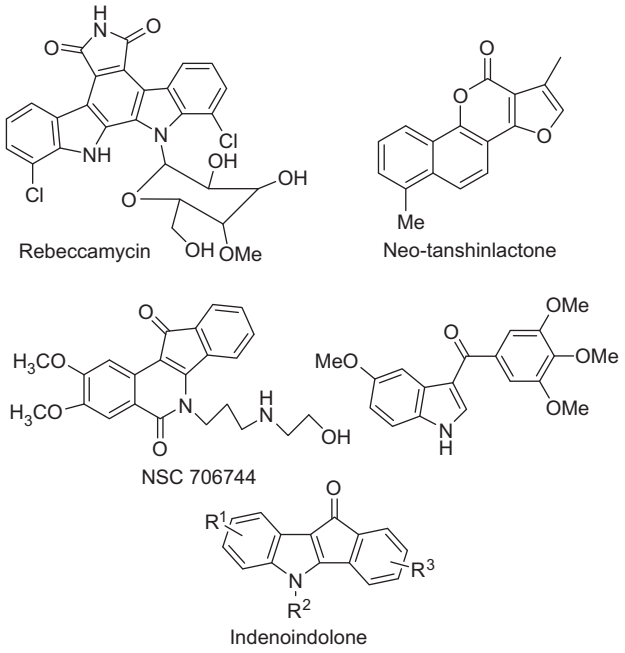


Figure 1. Natural/synthetic anticancer agents.

This protocol afforded the convenient preparation of diverse substituted/functionalized indenoindolones from easily accessible starting materials in reduced number (two only) of reaction steps. Following this process, a number of suitably relevant substituted indenoindolones were prepared (Scheme 1; Fig. 2).

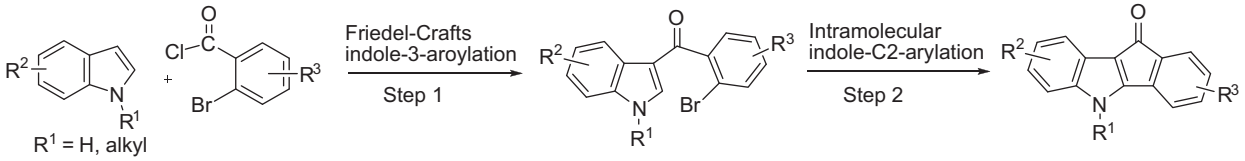
Cytotoxicity of all synthesized compounds (Fig. 2) was measured by using MTT assay in human embryonic kidney cancer cells (HEK 293, cat. # CRL-1573) and normal monkey kidney cells (Vero, cat. # CCL-81).[11,14,15] HEK 293 and Vero cells were purchased from American Tissue Culture Collection, VA, USA. According to DNA profiling and cytogenetic analysis, HEK 293 is hypotriploid and its nodal chromosome number is 64 occurring 30% of cells, but Vero cell is hypodiploid and its nodal chromosome number is 58 occurring 66% of cells. Approximately 10,000 cells were plated in a 96-well tissue culture plate and were treated with investigational compounds for 48 h. The cells were washed with 1X PBS and then MTT was added. It was incubated for 6 h at 37 °C. Detergent solution (10% NP-40 with 4 mM HCl) was added to each well of the culture plate and the color intensity was measured at 570 nm using the microplate reader (Berthold, Germany). Among all indenoindolones, compounds **2**, **5**, **6**, **10** and **14** showed relatively higher cytotoxic activities in comparison to others. With increasing concentration of these compounds, the cell death of HEK 293 significantly increased in comparison to Vero cells (Fig. 3). Compounds **2**, **5**, **6**, **10** and **14** caused 50% cell death (LC_{50}) of HEK 293 cells at 17, 17, 15, 15, and 22 μM, respectively. However, the LC_{50} values for Vero cells for compounds **2**, **5**, **6** and **10**, were found to be 53, 39, 48, and 54 μM, respectively. Interestingly, compound **14** even at 100 μM did not cause 50% cell death in Vero cells (Fig. 3). Etoposide, a widely used anticancer drug, showed LC_{50} at 18 μM for HEK 293 cells and at 28 μM for Vero cells. The LC_{50} values of all these five investigational compounds at various time periods in both the Vero and HEK 293 cell lines are depicted in Table 1. It was observed that anti-cell proliferative effect of tested indenoindolones did not change significantly after 48 h of treatment.

To compare the anti-proliferation potential of the investigational compounds with a clinically used common anticancer drug 5-FU, an experiment using HEK 293 cells was carried out (Fig. 4). The cells were treated with various concentrations of the investigational compounds and also that of 5-FU for 48 h and then the cell survivility was measured by MTT assay.[11,14,15] The LC_{50} values for the compounds **2**, **5**, **6**, **10** and **14** were 17, 17, 15, 15, and 22 μM, respectively, whereas 5-FU caused 50% cell death at 25 μM. Thus, these data indicate that the investigational compounds possess higher anti-cell proliferation potential than 5-FU in HEK 293 cells.

To determine the effect of compounds **2**, **5**, **6**, **10** and **14** on apoptosis in HEK 293 cells, DAPI nuclear staining was performed (Fig. 5A).[11,14,15] Cells when 70–80% confluent, were treated with investigational indenoindolones for 48 h. After treatment cells were washed with 1X PBS and fixed with acetone–methanol (1:1) and then DAPI reagent was added. Finally, images were taken under fluorescence microscope (Nikon, Japan) at 40× magnification. It was observed that the punchate, bubble shape, shrinkage (apoptotic) nuclei increased in HEK 293 cells on treatment with investigational compounds at their respective LC_{50} concentrations (Fig. 5A). The relative number of apoptotic/non-apoptotic nuclei generated in HEK 293 cells is presented as bar diagram in Figure 5B (P <0.05).

The regulation of cell cycle profile was investigated for understanding the mechanism by which these compounds can induce apoptosis. The kidney cancer cells after treatment with compounds (with respective LC_{50} concentrations) for 48 h were studied using FACS by staining with propidium iodide (Fig. 5C).[11,14,15] Cells were cultured in 60 mm tissue culture disc and were treated with the investigational compounds for 48 h. After the end of the treatment, cells were washed with PBS containing RNase-A. The cells were then fixed with ethanol and incubated at −20 °C overnight and later they were stained with PI. The cells were then sorted by FACS (Becton and Dickinson, CA) and DNA content of the cells at various phases of the cell cycle was measured by Cell Quest Software (Becton and Dickinson, CA). The effect of compounds **2**, **5**, **6**, **10** and **14** resulted in the accumulation of cells at G2/M phase of cell cycle. The exact biological target(s) for the anticancer activities of these indenoindolones are yet unknown in this preliminary investigation. The possible targets can be those proteins responsible for G2/M phase of the cell cycle, such as different cyclins, cyclin dependent kinase (CDKs) families, p21 and p53.

To further confirm the apoptotic effect of these five investigational compounds inside the cell, a western blot analysis with



Scheme 1. Reaction conditions[13b]: For step 1; indole (1.3 mmol), 2-bromobenzoyl chloride (1 mmol), $ZrCl_4$ (1.5 mmol), DCE, addition at 0 °C, 0 to 30 °C, then continuation at 30 °C. For step 2; compounds **1**–**5**: NH-protected 3-aroylindole (1 mmol), $Pd(dppf)_2Cl_2{\cdot}CH_2Cl_2$ (5 mol %), K_2CO_3 (2 equiv), DMF, 130 °C; For step 2; compounds **6**–**15**: NH-unprotected 3-aroylindole (1 mmol), $Pd(OAc)_2$ (5 mol %), Ph_3P (10 mol %), CsOAc (2 equiv), DMA, 130 °C.

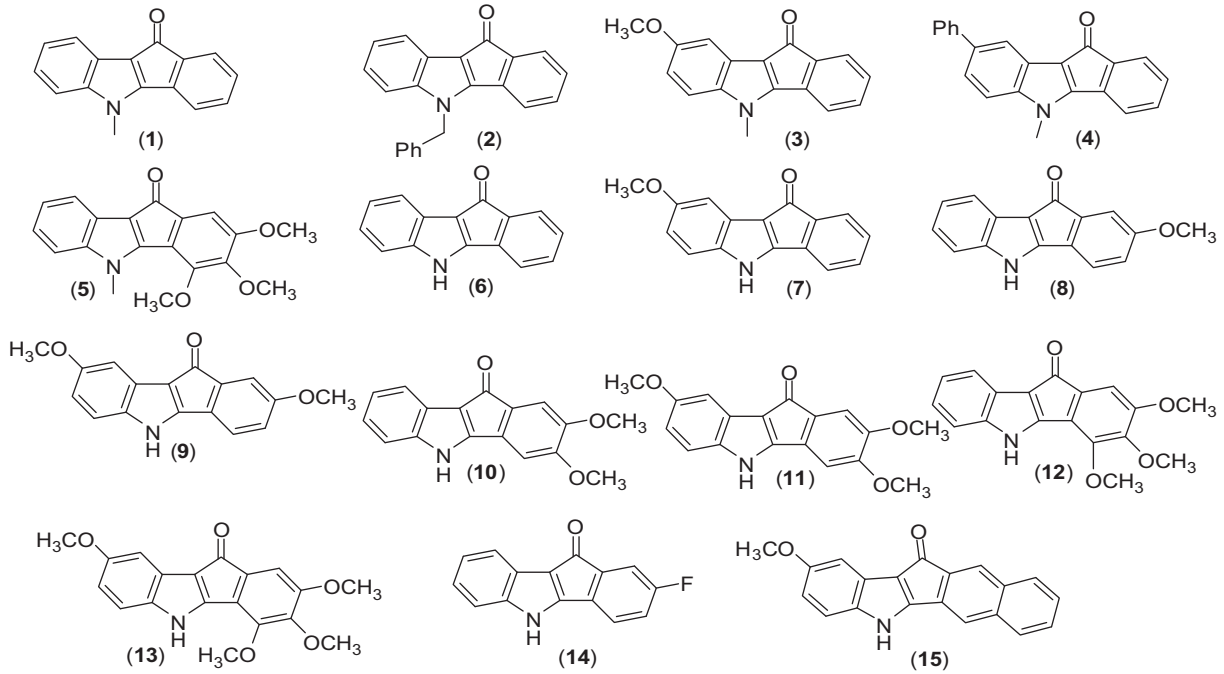


Figure 2. Synthesized indenoindolones.

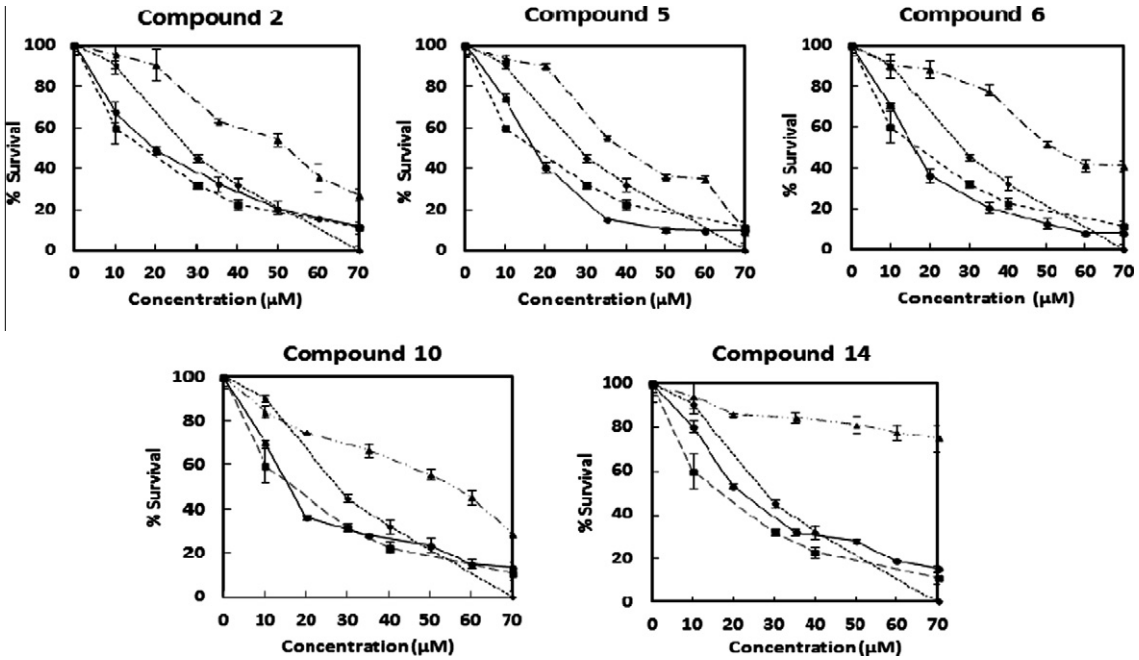


Figure 3. MTT cell survival assay. Cells were treated with investigational compounds and etoposide of various concentrations for 48 h and the experiment was carried out according to the known protocol.[11,14,15] The sign —♦—, —■—, –·–▲–·– and —●— represent for Vero/etoposide, HEK 293/etoposide, Vero/investigational compound and HEK 293/investigational compound, respectively. Data is the mean ± SD of three different experiments.

Table 1
LC_{50} values of the investigational compounds at different time periods

Compound	24 h		48 h		72 h	
	VERO (μM)	HEK 293 (μM)	VERO (μM)	HEK 293 (μM)	VERO (μM)	HEK 293 (μM)
2	57 ± 2	50 ± 2	53 ± 2	17 ± 2	45 ± 2	16 ± 2
5	42 ± 2	39 ± 2	39 ± 2	17 ± 2	35 ± 2	16 ± 2
6	56 ± 2	50 ± 2	48 ± 2	15 ± 2	40 ± 2	14 ± 2
10	47 ± 2	16 ± 2	54 ± 2	15 ± 2	55 ± 2	12 ± 2
14	>100	18 ± 2	>100	22 ± 2	65 ± 2	15 ± 2

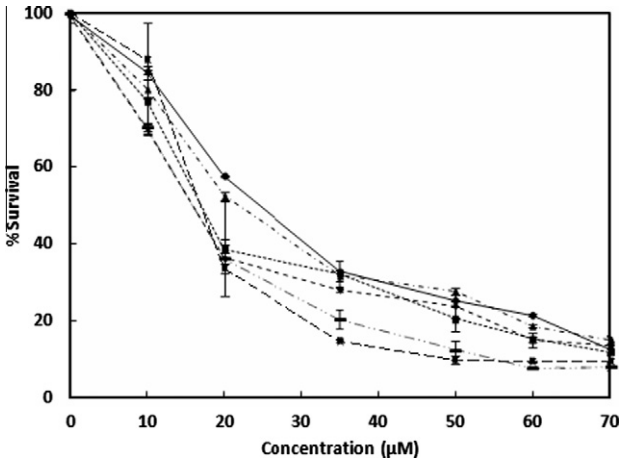


Figure 4. Comparison of cell survival activities of compounds (**2**, **5**, **6**, **10**, and **14**) with 5-FU in HEK 293 cells by MTT assay. Data is the mean ± SD of three different experiments. The symbols —●—, —■—, – – –– – –, –· –··, —♦—, and –·–▲–·– represent 5-FU, compounds **2**, **5**, **6**, **10** and **14**, respectively.

apoptotic markers was carried out (Fig. 6).[11,14,15] The level of cleaved product of PARP (86 KD) was higher in comparison to control when treated with the investigational compounds for 48 h. The expression of BCL-XL reduced compared to control. However, in contrast to BCL-XL, the expression level of BAX increased. The cleaved products of Caspase 3 (antibody cat #9962, cell signaling, CA, USA) increased in comparison to control. The level of housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured to confirm the same amount of protein loaded in each well. The increased levels of BAX/BCL-XL ratio, and cleaved product of Caspase 3 and PARP indicate that the cells underwent apoptosis when exposed to these investigational compounds (**2**, **5**, **6**, **10** and **14**).

Experiment for studying the expression of Caspase 3 level was also done by immunocytochemistry analysis of HEK 293 cells after exposure to investigational compounds by Caspase 3 antibody (cat #9662, cell signaling) (Fig. 7).[16,17] The images showed the increased accumulation of Caspase 3 protein expression in treated cells compared to control. All together, these results indicate that the investigational compounds caused apoptosis in HEK 293 cells.

In conclusion, the indenoindolones have been found to possess anticancer activities with higher potency compared to etoposide and 5-FU in kidney cancer cells and low toxicity to normal cells. The anticancer activities of these compounds were due to their apoptotic effect with cell cycle arrest at G2/M phase. These studies indicate that this class of compounds with further studies of biochemical pathways and related structural modulation by facile diversity-feasible synthesis may find their potential applications in anticancer drug discovery.

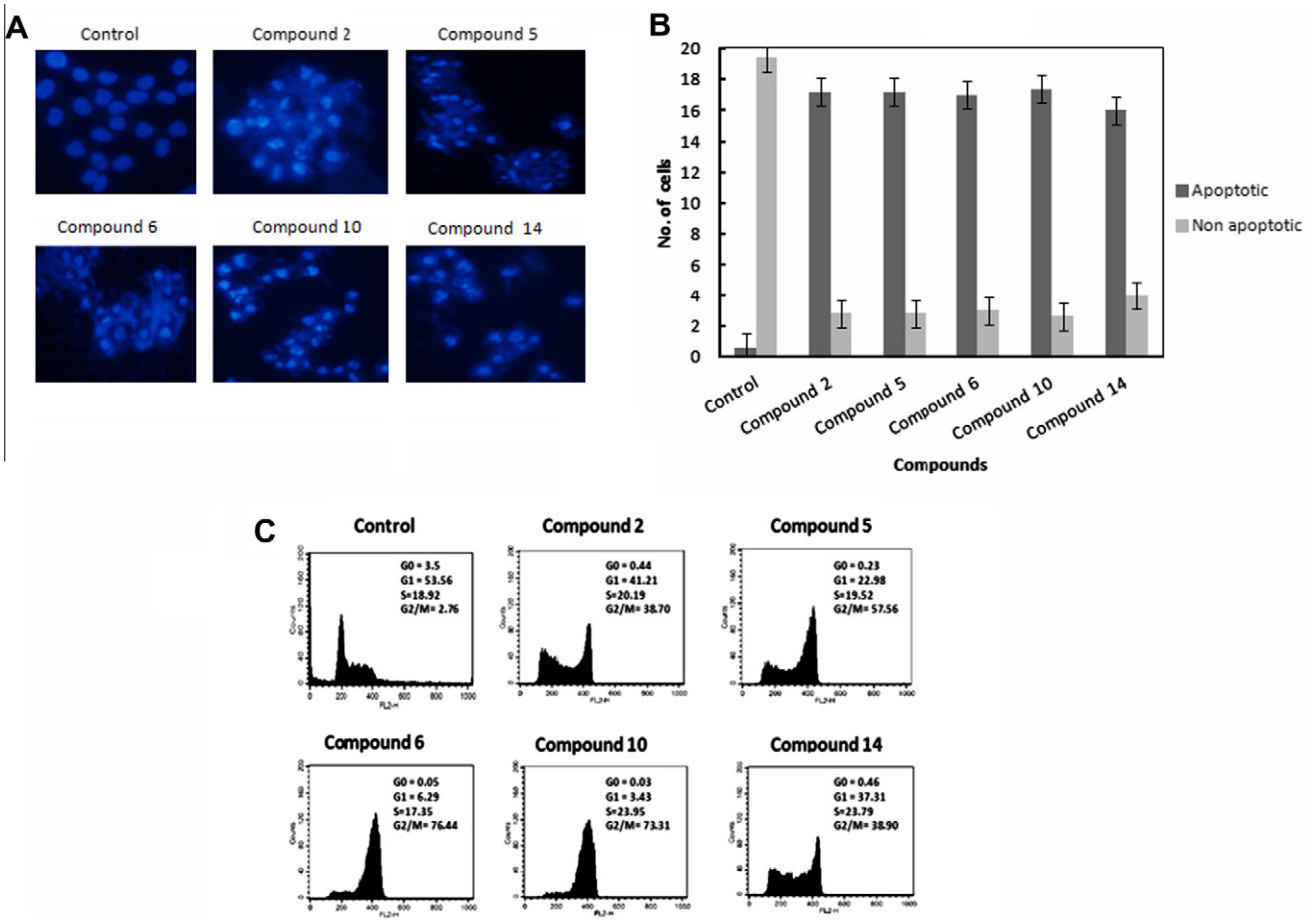


Figure 5. (A) DAPI nuclear staining. HEK 293 cells were treated with indenoindenoindolones (**2**, **5**, **6**, **10** and **14**) of their LC_{50} concentrations. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at 40× magnification. Data is the representation of one of the replicates of three different experiments. (B) A graphical representation of apoptotic nuclei of (A) (P <0.05). (C) FACS analysis was done and then the DNA content of the cell was measured by Cell Quest Software (Becton and Dickinson, CA).

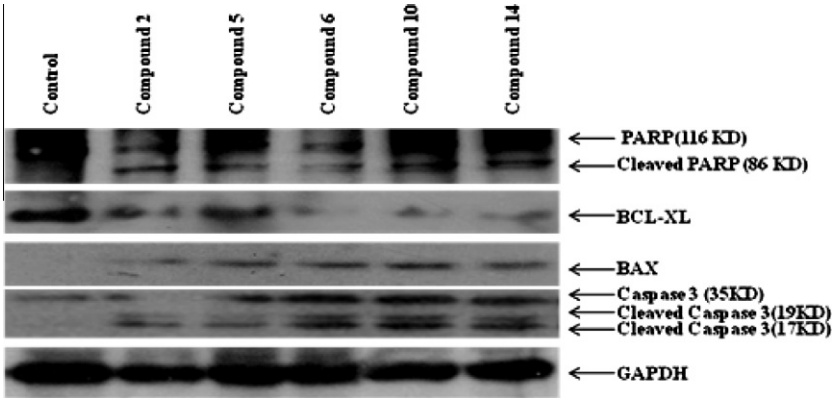


Figure 6. Western blot analysis with effect of indenoindolones on apoptotic markers in HEK 293 cells after treated for 48 h. Lower panel (GAPDH) represent the equal loading of proteins in each lane.

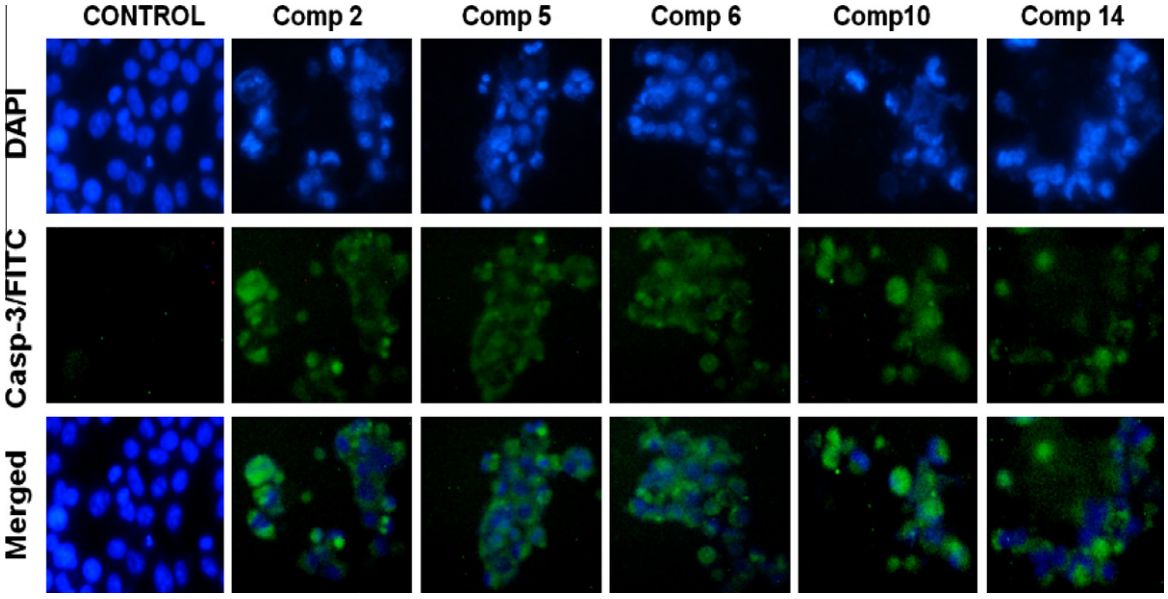


Figure 7. Immunocytochemistry analysis of Caspase 3 expression: HEK 293 cells were treated with the investigational compounds for 48 h. After their fixation and permeabilization, the nuclei were stained with DAPI (upper panel) followed by incubation with caspase 3 antibodies (cat #9662 from cell signaling) and FITC labeled secondary antibodies (middle panel). The photographs were taken of the same fields in both the upper and middle panels. Lower panel represents the merged images of FITC and DAPI staining.

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