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Synthesis, anticancer activity and apoptosis inducing ability of bisindole linked pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates

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ABSTRACT

A series of bisindole–pyrrolobenzodiazepine conjugates (**5a**–**f**) linked through different alkane spacers was prepared and evaluated for their anticancer activity. All compounds exhibited significant anticancer potency and the most potent compounds **5b** and **5e** were taken up for detailed studies on MCF-7 cell line. Cell cycle effects were examined apart from investigating the inhibition of tubulin polymerization for compounds **2a**, **2b**, **5b** and **5e** at 2 μM. FACS analysis showed that at higher concentrations (4 and 8 μM) there was an increase of sub-G1 phase cells and decrease of G2/M phase cells, thus indicating that compounds **5b** and **5e** are effective in causing apoptosis in MCF-7 cells. It was also observed that compounds **5b** and **5e** showed the down regulation of histone deacetylase protein levels such as HDAC1, 2, 3, 8 and increase in the levels of p21, followed by apoptotic cell death. The apoptotic nature of these compounds was further evidenced by increased expression of cleaved-PARP and active caspase-7 in MCF-7 cells.

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Indole derivatives have been found to exhibit anticancer activity by interacting with different intracellular targets. Naturally occurring as well as synthetic indolocarbazoles indicated inhibitory activity against cyclin-dependent kinases (CDKs) and antiproliferative activities in a variety of cell lines.[1,2] Indole-3-carbinol (I3C, **1**, Fig. 1), a dietary component found predominantly in cruciferous vegetables, is known to exhibit antiproliferative as well as apoptotic activities against various cancer cells,[3] and presently entered phase I and II clinical trials for breast cancer prevention.[4] Moreover, **1** itself does not possess anticancer activity, but due to the formation of active metabolites in the stomach by gastric juice mainly 3,3′-bisindolyl methane (BIM) a major metabolite may be responsible for its in vivo anticancer effects.[5] Recently, bisindolyl maleimides have been identified as NAD^+-dependent histone deacetylase (HDAC) inhibitors.[6] More recently, 1,1-bis(3′-indolyl)-1-(*p*-hydroxyphenyl)methane (**2b**) has been reported to exhibit antiproliferative and apoptotic effect in pancreatic cancer cells in vitro as well as in vivo.[7] Furthermore, bisindolyl methane moiety linked hydroxamic acids and related compounds act as HDAC inhibitors.[8,9] In addition, bisindoles and a number of indole derivatives are well known to possess potent anticancer activity.[10–14]

Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs), are an important class of sequence selective DNA-interactive agents that bind covalently to guanine bases within the minor groove of DNA.[15] Well-known members of this group[16,17] include anthramycin, tomaymycin, sibiromycin, limazepine and DC-81 (**3**). Recently, a PBD dimer (SJG-136) has entered the phase II clinical trial.[18] Although PBDs have shown high antitumour activity, significant cardiotoxicity hampers their clinical applications.[19] Accordingly, there has been considerable interest in the design of various PBD conjugates by linking this scaffold to other DNA interactive agents with a view to enhance the anticancer activity while decreasing side effects.[20–23] Recently, Wang and co-workers synthesized indole-PBD conjugates (**4**) exhibiting anticancer activity by inducing apoptosis through mitochondrial mediated pathway.[24]

Furthermore, we have not only been involved in the development of new synthetic strategies[25] for the preparation of PBD ring system but also in the design of structurally modified PBDs and their hybrids.[26–28] In continuation to our efforts in search of potent molecules that exhibit anticancer activity,[29–31] and based on the interesting biological activities of bisindoles, we designed and synthesized bisindole linked PBD's (**5**) to explore their potential as anticancer agents. It is expected that combination of two active pharmacophores with in a single molecule would enhance the anticancer activity by an additive or synergistic effect. Therefore, we coupled an active moiety of known antitumour bisindolyl methane derivative (**2**) through their 3- and 4-hydroxyl groups

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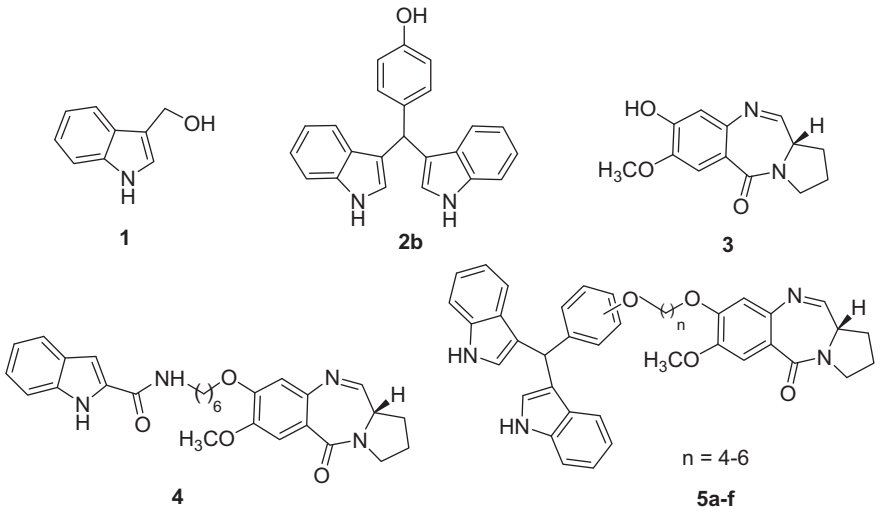


Figure 1. Chemical structures of indole-3-carbinol (**1**), bisindole (**2b**), DC-81 (**3**), indole-PBD conjugate (**4**) bisindole–PBD conjugates (**5a**–**f**).

to **3** via different alkyl spacers (Fig. 2). The in vitro cytotoxicity and other biological investigations of these conjugates on MCF-7 cell line in regulating HDACs as well as CDK inhibitor p21 were carried out.

The preparation of bisindole intermediates (**2a,b**) was accomplished by following a novel method recently developed in our laboratory.[32] Reaction of substituted hydroxy benzaldehydes (**7a,b**) with indole in acetonitrile using aluminium triflate as a catalyst afford the phenol substituted derivatives (**2a,b**) as illustrated in Scheme 1. The synthesis of C8-linked bisindole–PBD conjugates (**5a–f**) was carried out from the (2*S*)-*N*-{4-[3-bromoalkoxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde-diethylthioacetal (**8**), which has been prepared by the methods reported in our earlier studies[33–36] and phenolic bisindole precursors (**2a,b**) using K_2CO_3 in acetone provided the nitro thioacetal intermediates **9a–f**. Finally, these upon reduction with $SnCl_2{\cdot}2H_2O$ in refluxing methanol followed by thiol deprotection by $HgCl_2/CaCO_3$ in $CH_3CN–H_2O$ at room temperature afforded the desired PBD conjugates **5a–f**.[37]

These conjugates **5a–f** were evaluated for their anticancer activity in selected human cancer cell lines of lung, breast, oral, colon, prostate and ovary by using sulforhodamine B (SRB) method. All these compounds **5a–f** exhibited anticancer potency with GI_{50} values ranging from 0.11 to 30.8 μM, whereas the positive controls adriamycin[38] and DC-81 demonstrated GI_{50} values in the range of <0.1−14.7 μM and 0.10–2.37 μM concentrations, respectively. Interestingly all the compounds are active in human breast cancer cell line (MCF-7) with GI_{50} values ranging from 0.14 to 2.01 μM (Table 1). Compounds **5b** and **5e** in which the PBD moiety was linked, to bisindole substituted phenolic group by odd number of alkyl spacers ($n = 3$) exhibit better anticancer potency than their counterparts with even number of decreased alkyl spacer length (**5a** and **5d**; $n = 2$) and increased in the length of alkyl spacer (**5c** and **5f**; $n = 4$). All the synthesized compounds have displayed significant anticancer potency against breast cancer cell line (MCF-7) with GI_{50} values in the range of 0.14–2.01 μM.

With a view to understand the cytotoxic nature of some of the active compounds, MTT cytotoxicity assay was performed by

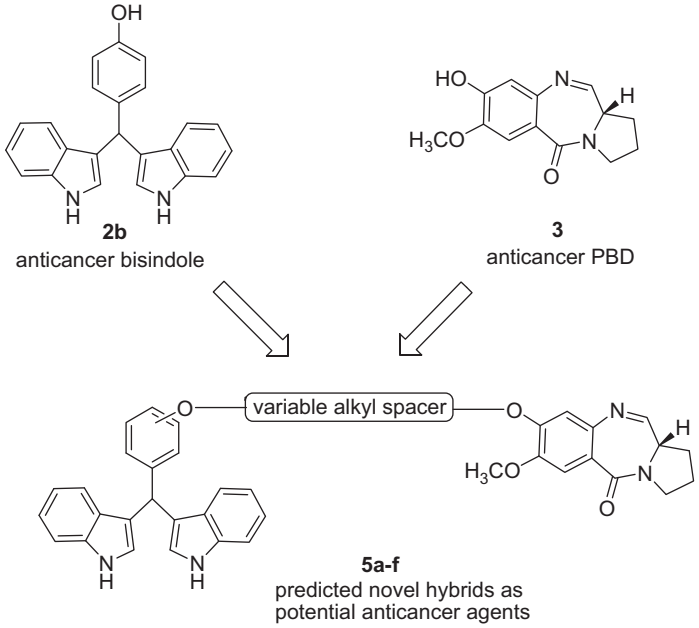
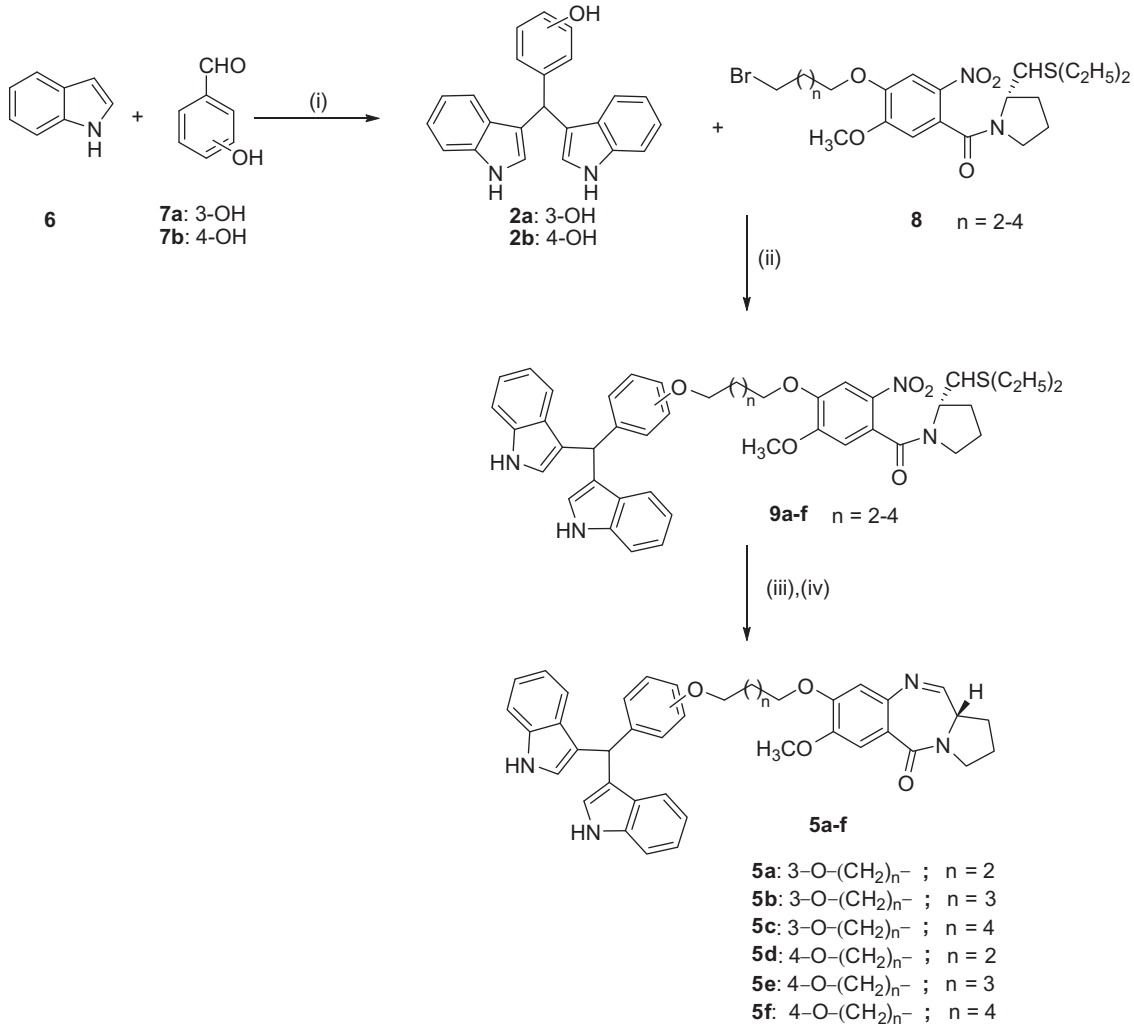


Figure 2. Design of bisindole–PBD conjugates (**5a**–**f**) as potential anticancer agents.



Scheme 1. Reagents and conditions: (i) $Al(OTf)_3$, dry acetonitrile, rt, 1–2 h, 93–95%; (ii) K_2CO_3, acetone, reflux, 24 h, 84–88%; (iii) $SnCl_2{\cdot}2H_2O{\cdot}MeOH$, reflux, 3 h, 89–95%; (iv) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O (4:1) rt, 8 h, 40–52%.

Table 1
Anticancer activity (GI_{50} values in μM)[a] for compounds **5a–f** in selected human cancer cell lines

Code	MCF-7[b]	Zr-75-1[b]	A2780[c]	Colo205[d]	SiHa[e]	A549[f]	KB[g]
5a	2.01	—[h]	27.00	28.50	—[h]	—[h]	—[h]
5b	0.19	—[h]	0.18	0.16	2.20	—[h]	2.30
5c	2.01	—[h]	2.29	2.30	30.8	—[h]	30.1
5d	0.18	26.50	2.07	24.0	2.49	—[h]	2.30
5e	0.14	0.12	0.11	26.1	0.18	2.40	0.13
5f	0.14	—[h]	2.46	—[h]	—[h]	29.0	—[h]
DC-81	0.17	2.37	0.14	0.11	0.16	0.16	0.17
ADR	<0.1	<0.1	<0.1	14.7	1.90	13.01	0.16

[a] 50% Growth inhibition and the values are mean of three determinations.
[b] Breast cancer.
[c] Ovarian cancer.
[d] Colon cancer.
[e] Cervix cancer.
[f] Lung cancer.
[g] Oral cancer.
[h] GI_{50} values not attained at the concentrations used. ADR stands for adriamycin.

treating MCF-7 cells with compounds **3**, **2a**, **2b**, **5b** and **5e** at different concentrations ranging from 2 to 8 μM for 24 h. It is observed that compounds **5b** and **5e** cause almost 50% of cell death at 2 μM concentrations. Further these compounds are slightly higher cytotoxic than the positive control DC-81 (**3**), **2a** and **2b** (Fig. 3).

To investigate the effect of bisindole–PBD conjugates on the cell cycle progression of human breast cancer cell line (MCF-7), the DNA content of the cell nuclei was measured by flow cytometric analysis. MCF-7 cells were treated with compounds **3**, **2a**, **2b**, **5b** and **5e** at 2 μM concentration which resulted in accumulation of

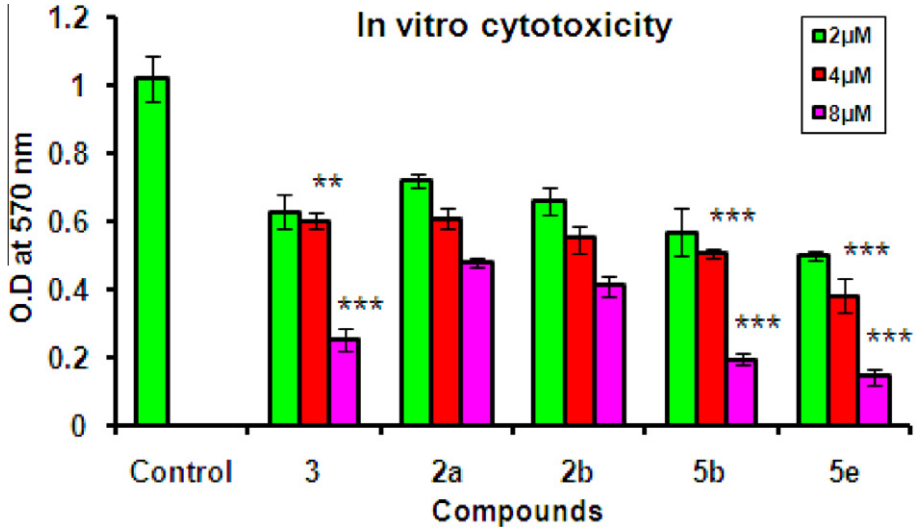


Figure 3. The in vitro cytotoxicity assay for bisindole–PBD conjugates. The MCF-7 cells were treated with various concentrations of compounds **3**, **2a**, **2b**, **5b** and **5e** and MTT assay was conducted which reveals the viable cells present after compound treatment. The values were found to be statistically significant with $^{**}p$ <0.01, $^{***}p$ <0.001 when compared to control untreated cells. Error bars represent the mean ± standard deviation (SD) of at least three measurements.

Table 2
Cell cycle distribution of MCF-7 cell line at different concentrations (2, 4 and 8 μM of compounds DC-81 (**3**), **2a**, **2b**, **5b**, and **5e**

Compound	Conc. (μM)	G0	G1	S	G2/M
Control	2	3.03 ± 0.15	47.01 ± 1.00	15.25 ± 0.48	34.71 ± 1.51
3	2	3.14 ± 0.52	55.11 ± 1.55	8.02 ± 0.23	33.79 ± 0.73
2a	2	2.80 ± 0.26	51.23 ± 1.12	9.86 ± 0.32	36.03 ± 0.95
2b	2	3.16 ± 0.28	53.85 ± 0.79	9.26 ± 0.65	33.7 ± 0.25
5b	2	2.98 ± 0.48	38.86 ± 0.74	15.42 ± 0.76	42.67 ± 1.34
5e	2	3.57 ± 0.35	40.67 ± 0.76	9.08 ± 0.22	46.67 ± 1.34
Control	4	3.11 ± 0.11	65.60 ± 0.61	5.90 ± 0.32	25.38 ± 0.78
3	4	9.27 ± 0.28	70.84 ± 1.23	8.56 ± 0.87	11.31 ± 0.30
2a	4	3.63 ± 0.63	50.56 ± 0.51	9.41 ± 0.52	36.3 ± 0.59
2b	4	5.01 ± 1.02	49.65 ± 1.51	9.78 ± 1.07	35.56 ± 0.75
5b	4	10.70 ± 0.3	70.29 ± 1.44	8.26 ± 0.35	10.74 ± 1.50
5e	4	16.11 ± 0.3	68.33 ± 0.39	6.95 ± 0.06	8.60 ± 0.45
Control	8	3.11 ± 0.11	65.60 ± 0.61	5.90 ± 0.32	25.38 ± 0.78
3	8	29.68 ± 0.59	57.74 ± 0.65	4.53 ± 0.50	8.05 ± 0.92
2a	8	6.03 ± 0.11	45.99 ± 0.99	10.02 ± 0.96	37.97 ± 1.01
2b	8	7.05 ± 0.99	46.19 ± 1.30	8.99 ± 0.017	37.97 ± 1.29
5b	8	24.81 ± 0.50	60.92 ± 0.81	5.26 ± 0.47	8.99 ± 1.04
5e	8	50.15 ± 1.73	46.11 ± 1.87	2.32 ± 0.24	1.41 ± 0.32

34%, 36%, 33%, 42% and 45% of cells in G2/M phase, when compared to untreated cells. Further treatment of these compounds at higher concentrations (4 and 8 μM) for 24 h resulted in larger accumulation of sub-G1 populated cells with a concomitant decrease in the G2/M phase especially in compounds **3, 5b** and **5e**. However, the intermediates **2a** and **2b** have shown only G2/M phase arrest at all the concentrations (**2**, **4** and **8** μM) (Table 2). Therefore increase of cells in sub-G1 clearly showed that these hybrid compounds are effective in causing apoptosis in MCF-7 cells as shown in (Figs. 4a and 4b). Similar observations were previously found for the indole-PBD conjugate (IN6CPBD).[24]

Generally, inhibition of tubulin polymerization was associated with cell cycle arrest at G2/M phase transition by interrupting mitotic spindle formation.[39] From the previous reports, it is well known that molecules that alter the microtubule polymerization, cause mitotic arrest which ultimately leads to apoptosis.[40] Thus the effect of these compounds **3**, **2a, 2b, 5b** and **5e** on the tubulin cytoskeleton and its integrity was examined at 2 μM by taking nocodazole as the standard. The cells were fixed and stained after 24 h with FITC conjugated α-tubulin antibody. Interestingly, disruption of tubulin polymerization was observed in this assay, thus confirming cell cycle arrest at G2/M phase (Fig. 5). DC-81 (**3**), the standard PBD molecule did not show any effect on tubulin as it causes G1 cell cycle arrest (Supplementary data Fig. S1).

Histones are the nuclear core proteins that regulate transcription and cell cycle progression[41] and inhibition of HDACs is a promising novel strategy in human cancer therapy.[42] HDAC depletion is known to activate CDK inhibitors p21 as well as p27 and trigger the G2/M cell cycle arrest.[43] To understand the molecular events involved in G2/M phase arrest, the effect on the expression levels of histones and CDK inhibitor p21, which plays a vital role in cell cycle progression was also investigated in MCF-7 cells. Thus MCF-7 cells were treated with compounds trichostatin A (TSA, the standard HDAC inhibitor), **3**, **2a**, **2b**, **5b** and **5e** at 2 μM concentrations for 24 h and cell lysates were collected, subjected to Western blotting. Results indicate that down regulation of HDACs protein levels such as HDAC1, 2, 3, 8 were observed when compared to the untreated control cells. Moreover compounds **5b** and **5e** selectively induced significant increase in the protein levels of p21 and similar observations were found in the study response of estrogen receptor (ER)-positive MCF-7 cells and effect of HDAC inhibitors[44] (Figs. 6a, 6b and 6c).

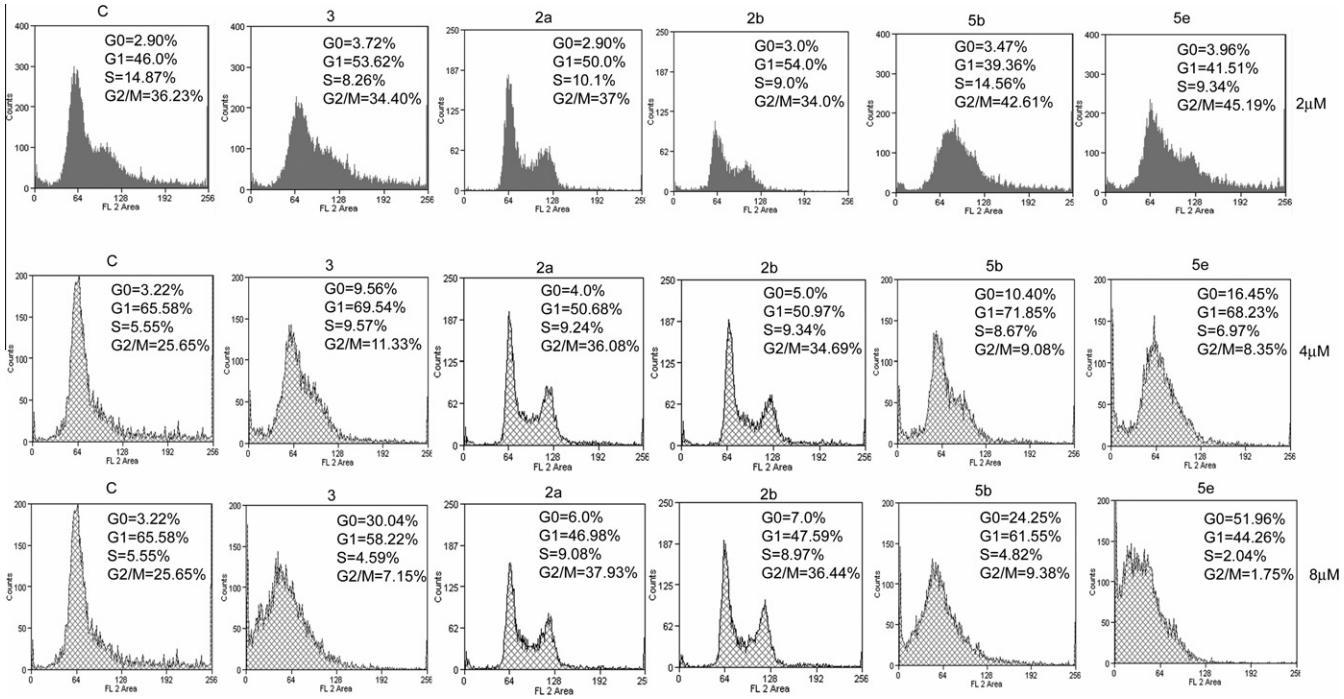


Figure 4a. Effect of bisindole–PBD conjugates on cell cycle. MCF-7 cells were treated with compounds **3**, **2a**, **2b**, **5b** and **5e** at various concentrations **2**, **4** and **8** μM for 24 h. At 2 μM the cells showed G2/M cell cycle arrest and increased concentration caused accumulation of more cells in sub G1, indicating apoptosis, DC-81(**3**) is the positive control. Whereas starting material **2a** and **2b** continued to show G2/M phase arrest at 2, 4 and 8 μM. One of three independent experiment values have been depicted.

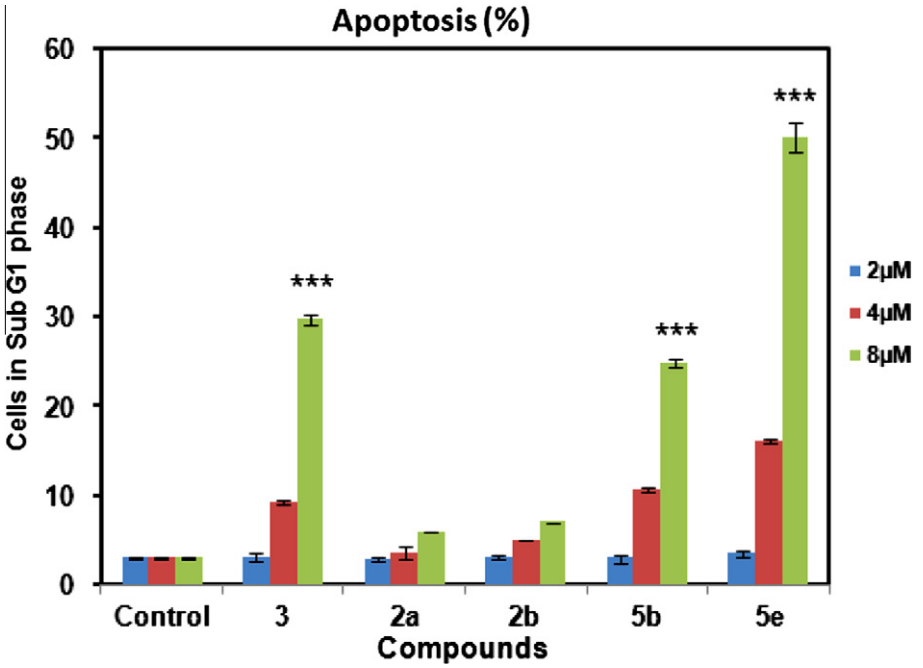


Figure 4b. The graph depicting the percentage of apoptosis after treatment of compounds **3**, **2a**, **2b**, **5b** and **5e** at 2, 4 and 8 μM concentrations for 24 h. The values were found to be statistically significant with $^{***}p$ <0.001 when compared to control untreated cells. Error bars represent the mean ± SD of at least three measurements.

Previous studies by Twiddy et al.[45] have discovered that caspase-7 and PARP play an important role in causing apoptosis in human breast cancer cells. To prove whether these conjugates cause apoptosis, MCF-7 cells were treated with compounds **3**, **2a**, **2b**, **5b** and **5e** at 8 μM concentration and cell lysates were subjected to Western analysis. A signal for cleaved-PARP and activated capase-7 was detected in MCF-7 samples treated with these compounds (8 μM, 24 h) (Fig. 7). This finding confirms the apoptosis inducing nature of these compounds.

The synthesis and evaluation of anticancer activity of a new series of bisindole–PBD conjugates has been investigated. Most of the compounds have exhibited potent anticancer activity against selected human cancer cell lines. Cell cycle effects were examined apart from investigating the inhibition of tubulin polymerization

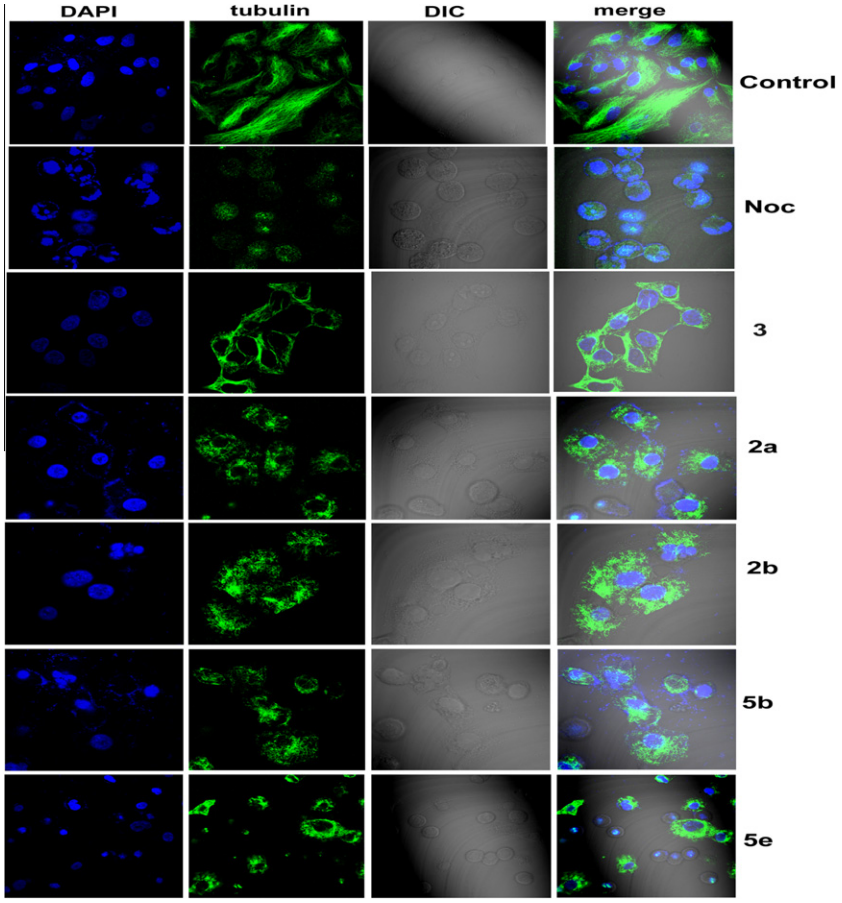


Figure 5. Effect of bisindole–PBD conjugates on tubulin polymerization. MCF-7 cells were exposed to **3**, **2a**, **2b**, **5b**, **5e**, and nocodazole at 2 μM concentration for 24 h and followed by tubulin staining. 'Noc' represents nocodazole, used as a positive control.

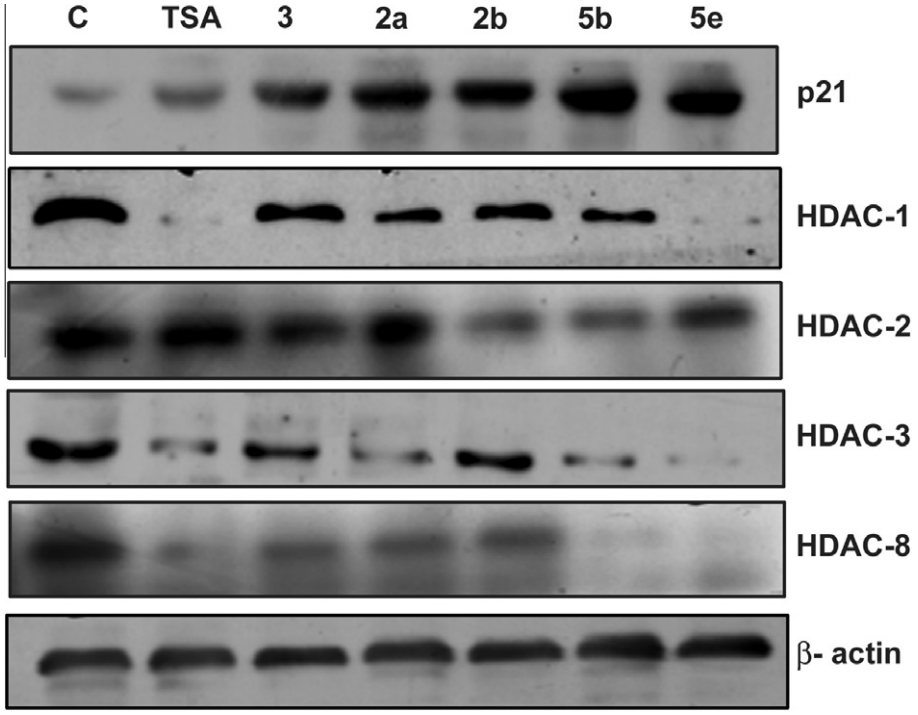


Figure 6a. Effect of bisindole–PBD conjugates on various proteins involved on class I HDACs and p21. MCF-7 cells were treated with compounds TSA, **3**, **2a**, **2b**, **5b** and **5e** for 24 h and cell lysates were subjected to Western blotting for p21, HDAC1, 2, 3 and 8. β-Actin used as a loading control.

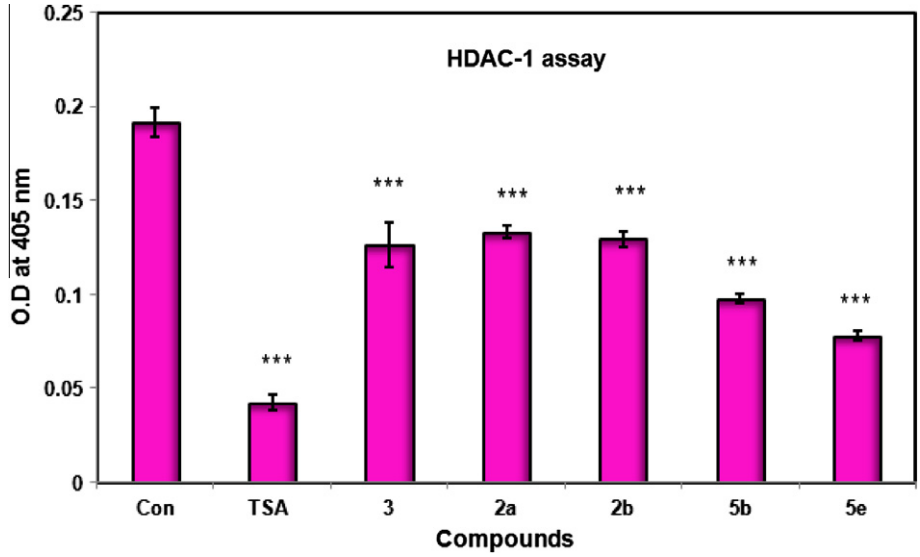


Figure 6b. Effect of bisindole–PBD conjugates on HDAC-1 protein. Compounds **TSA**, **3**, **2a**, **2b**, **5b** and **5e** were subjected to HDAC-1 photometric assay (Enzo Kit). Here TSA was used as standard chemical. The lesser O.D indicates the higher HDAC inhibitory activity. The values were found to be statistically significant with $^{***}p$ <0.001 when compared to control untreated cells. Error bars represent the mean ± SD of at least three measurements.

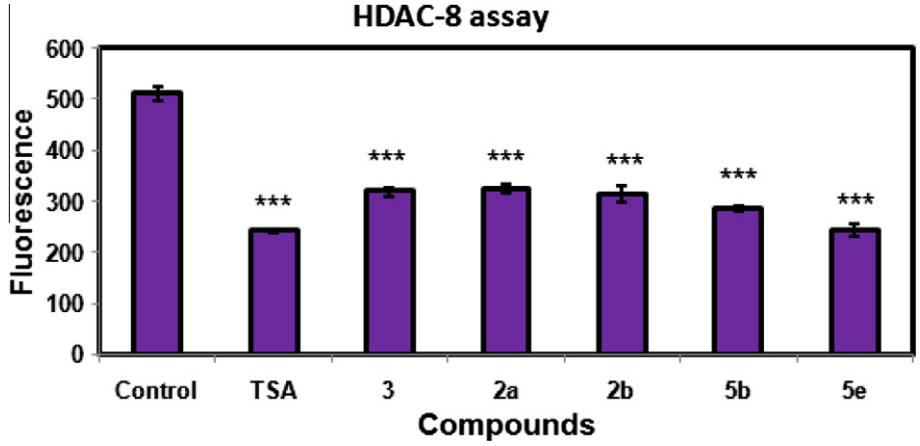


Figure 6c. Effect of bisindole–PBD conjugates on HDAC-8 protein. Compounds **TSA**, **3**, **2a**, **2b**, **5b** and **5e** were subjected to HDAC-8 fluorometry based assay (Enzo Kit). Here TSA was used as standard chemical. The lesser O.D indicates the higher HDAC inhibitory activity. The values were found to be statistically significant with $^{***}p$ <0.001 when compared to control untreated cells. Error bars represent the mean ± SD of at least three measurements.

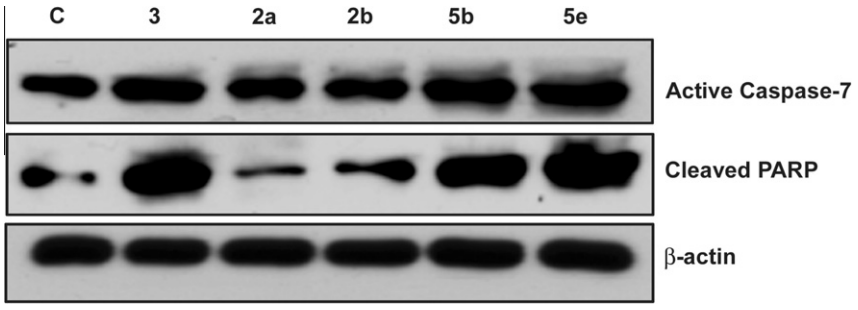


Figure 7. Effect of compounds on apoptosis, MCF-7 cells were treated with compounds **3**, **2a**, **2b**, **5b** and **5e** at 8 μM concentrations for 24 h. The cells were then processed and lysates were subjected to Western blotting analysis using antibodies against active caspase-7 and cleaved-PARP which are indicators of apoptosis. β-Actin used as a loading control. Results shown are indicative of three individual experiments.

for compounds **3**,**2a, 2b, 5b** and **5e**. Thus, we provided the evidence that these newly synthesized hybrid molecules inhibited growth of human breast carcinoma MCF-7 cells by inducing apoptosis. The apoptotic death was associated with down regulation of histone protein levels such as HDAC1, 2, 3, 8 and increased levels of p21. Further HDAC reduction was associated with an initiation of DNA damage that triggered apoptosis followed by cell death. In addition, we have observed increased expression of cleaved-PARP and

active caspase-7 proteins in MCF-7 cells. Further studies on biochemical changes and molecular events involved in cell death caused by these hybrids are underway.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.080.

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37. *Spectral data for (S)-8-(5-(3-(di(1H-indol-3-yl)methyl)phenoxy)pentyloxy)-7-methoxy-2,3-dihydro-1H-benzo[e] pyrrolo[1,2-a][1,4]diazepin-5(11aH)-one (**5b**)*: Yield 51%; mp 123–125 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.05 (br s, 2H), 7.66 (s, 1H), 7.51 (d, J = 4.1 Hz, 1H), 7.38 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 7.23–7.12 (m, 3H), 7.07–6.86 (m, 4H), 6.85 (s, 1H), 6.80-6.71 (m, 1H), 6.66 (d, J = 1.9 Hz, 2H), 5.88 (s, 1H), 4.09–3.88 (m, 4H), 3.85 (s, 3H), 3.84–3.66 (m, 3H), 2.01–1.80 (m, 6 H). 1.78–1.67 (m, 4H); MS (LC) m/z 653 $(M+1)^+$; IR (KBr) (ν_{max}/cm^{-1}): 3407, 2936, 2874, 1604, 1487, 1433, 1264, 1220, 1017, 875, 743; *(S)-8-(5-(4-(di(1H-indol-3-yl)methyl) phenoxy)pentyloxy)-7-methoxy-2,3-dihydro-1H-benzo[e]pyrrolo[1,2-a] [1,4]diazepin-5(11aH)-one (**5e**)*: Yield 49%; mp 127–129 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.99 (br s, 2H), 7.64 (s, 1H), 7.51 (d, J = 4.0 Hz, 1H), 7.42–7.34 (m, 4H), 7.23 (dd, J = 6.6, 1.9 Hz, 2H), 7.15 (dt, J = 6.9, 0.9 Hz, 2H), 6.99 (dt, J = 6.9, 0.9 Hz, 2H), 6.85–6.76 (m, 3H), 6.67–6.63 (m, 2H), 5.83 (s, 1H), 4.12–4.05 (m, 2H), 4.04–3.92 (m, 2H), 3.91 (s, 3H), 3.87–3.71 (m, 3H), 2.00–1.72 (m, 6H), 1.63–1.47 (m, 4H); MS (LC) m/z 653 $(M+1)^+$; IR (KBr) (ν_{max}/cm^{-1}): 3393, 2939, 1605, 1508, 1459, 1224, 1175, 1015, 746. The detail spectral data of other compounds are available in Supplementary data.
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