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Original article

Synthesis and potential cytotoxic activity of new phenanthrylphenol-pyrrolobenzodiazepines

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ABSTRACT

New phenanthrylphenol-pyrrolobenzodiazepine (PP-PBD) conjugates have been synthesized and evaluated for their biological activity. One of the compounds **4a** has been evaluated for its antiproliferative activity on 57 human tumour cell lines. The growth inhibition of **4a**–**c** has been determined by MTT viability assay on MCF-7 cell line. Among them, **4c** showed most potent growth inhibition. Based on this, an attempt was made to rationalize their mechanism of action through cell cycle analysis and DNA interaction studies. The effect of the lead compound **4c** on MCF-7 cell growth associated with cell cycle arrest in G1 phase, followed by apoptosis. Our findings suggested the phenanthrylphenol-PBD conjugate **4c**, which is a cyclin D1 inhibitor could be considered as a promising lead compound against breast cancer for further investigation.

1. Introduction

In spite of great progress in chemotherapy, there has been significant development in recent years in the number of new anticancer agents, with the emphasis on creating new DNA interactive drugs [1–3]. Presently, most of the anticancer drugs interact with DNA mainly by two binding modes, which are DNA minor groove binding through a combination of hydrophobic, electrostatic, hydrogen-bonding interactions and intercalative binding in which a planar aromatic moiety slides between the DNA base pairs [4–6]. A number of polycyclic aromatic hydrocarbons (PAHs) and their derivatives in view of their planar ring system are known to intercalate with DNA resulting in the anticancer activity [7,8]. PAHs have the potential to disrupt the normal function of cellular DNA and could lead to mutagenesis, carcinogenesis and even cell death [9,10]. A variety of phenanthrene ring systems intercalate with DNA, giving rise to many drugs that possess chemotherapeutic activity. As shown in Fig. 1 (**1** and **2**), substituted phenanthrenes with basic amino side chains have shown significant anticancer activity against breast cancer [11]. Similarly, the pyrrolo [9,10] phenanthrene molecules that effectively intercalate with DNA results in synergetic enhancement of DNA immobilization/cross linking [12]. Recently, several phenanthrene based tylophorine derivatives as potential anticancer agents have been reported [13]. These results together with other related studies indicate that a linear tricyclic chromophore is the minimum requirement for efficient intercalative binding [14].

On the other hand, it has been demonstrated that small molecules may recognize an increasing range of DNA sequences, which have DNA binding properties. These include pyrrolo [2,1-*c*][1,4]benzodiazepines (PBDs) and lexitropsins [15]. PBDs are well known naturally occurring DNA interactive antitumour antibiotics like anthramycin, chicamycin and DC-81 (**3**), which are produced from various *Streptomyces* bacteria [16]. They exert their biological activity through covalent binding via their N10–C11 imine/carbinolamine moiety to the C2-amino position of a guanine residue within the minor groove of duplex DNA. PBD monomers span three DNA base pairs with a preference for 5′-Pu-G-Pu, particularly 5′-AG or 5′-GA sequences [17]. The cytotoxicity of these agents is thought to be due to their ability to inhibit cellular processes such as replication and transcription.

Previously, in our laboratory a few hybrid molecules have been synthesized wherein chrysene and pyrene are linked to PBD moiety

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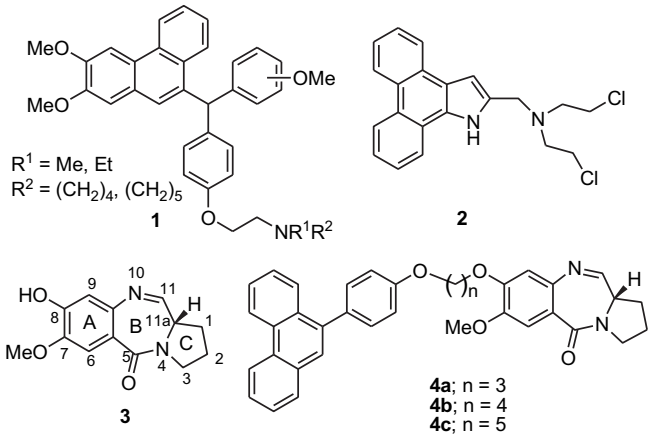


Fig. 1. Representative chemical structures of phenanthrenes (**1** and **2**), DC-81 (**3**), and phenanthrylphenol-PBD conjugates (**4a**–**c**).

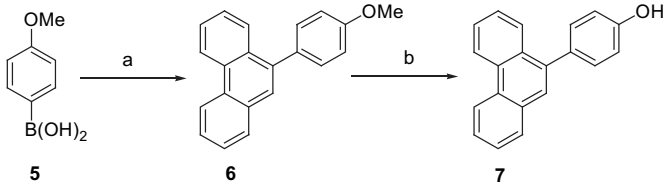
at C8 position [18,19]. We observed that, such hybrids of PBD that are linked to polycyclic aromatic hydrocarbons (PAH) enhance the anticancer activity compared to DC-81. Using similar design concepts, with the aim of obtaining new anticancer compounds we have synthesized new phenanthrylphenol linked PBD (PP-PBD) conjugates and evaluated the effect of these compounds on MCF-7 cell proliferation. Cell cycle arrest and apoptotic properties have also been evaluated. The reason for choosing MCF-7 cell line for our experimentation is that we have been interested in finding out the relationship between substituted phenanthrenes and breast anticancer activities as some of the phenanthrene derivatives are known to exhibit antiproliferative effect against breast cancer cells [11]. Moreover, the expression of cyclin D1 has shown to increase the proliferation rate of MCF-7 cells. We carried out our studies using this cell line since it has shown a GI_{50} value of 0.17 μM obtained from the National Cancer Institute (NCI) anticancer screening on the compound **4a**.

The results suggest that the synthetic PP-PBD is indeed a candidate for anticancer agent causing sub G1 arrest and apoptosis following the intrinsic mitochondrial death pathway as the mechanism of action. This proposed mechanism of intrinsic mitochondrial path is also consistent with the release of cytochrome c, activation of caspase-9 followed by PARP cleavage and decrease in cyclin D1 levels.

2. Results and discussion

2.1. Chemistry

Accordingly, Scheme 1 shows the synthesis of the required phenanthrylphenol (**7**) moiety for the preparation of DNA interactive PP-PBD conjugates. This synthetic strategy involves the use of Pd-catalyzed Suzuki cross coupling reaction for the arylation followed by demethylation. Starting material **6** has been obtained



Scheme 1. *Reagents and conditions*: (a) 9-bromophenanthrene, $Pd(PPh_3)_4$, K_3PO_4, dioxane–H_2O, 80 °C, 16 h, 70%; and (b) BBr_3, dry CH_2Cl_2, −78 °C, 5 h, 80%.

by cross coupling of commercially available 9-bromophenanthrene and 4-methoxyphenylboronic acid (**5**) using $Pd(PPh_3)_4$ and K_3PO_4 in 1,4-dioxane/H_2O. Then, demethylation step is carried out with borontribromide (BBr_3) in anhydrous CH_2Cl_2 to give the required phenathrylphenol intermediate **7**.

The key precursor **8** has been prepared from commercially available vanillin through several straightforward functional group transformations as reported in our earlier studies [20]. Then the debenzylation of intermediate **8** with $BF_3.OEt_2$/EtSH gives (2*S*)-*N*-((4-hydroxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal (**9**). Etherification of this hydroxyl compound **9** with various dibromoalkanes in the presence of K_2CO_3 produces the (2*S*)-N-{4-[3-bromoalkoxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal intermediates (**10a**–**c**). Later, the phenanthrylphenol precursor **7** is coupled to each of the intermediate **10** using K_2CO_3 in acetone to provide the nitrothioacetal intermediates **11a**–**c**. Finally, these upon reduction with $SnCl_2.2H_2O$ in methanol followed by deprotective-cyclization using $HgCl_2$–$CaCO_3$ affords the desired target PBD conjugates **4a**–**c** in good yields ranging from 58 to 60% as depicted in Scheme 2.

2.2. Evaluation of biological activity

2.2.1. Anticancer activity

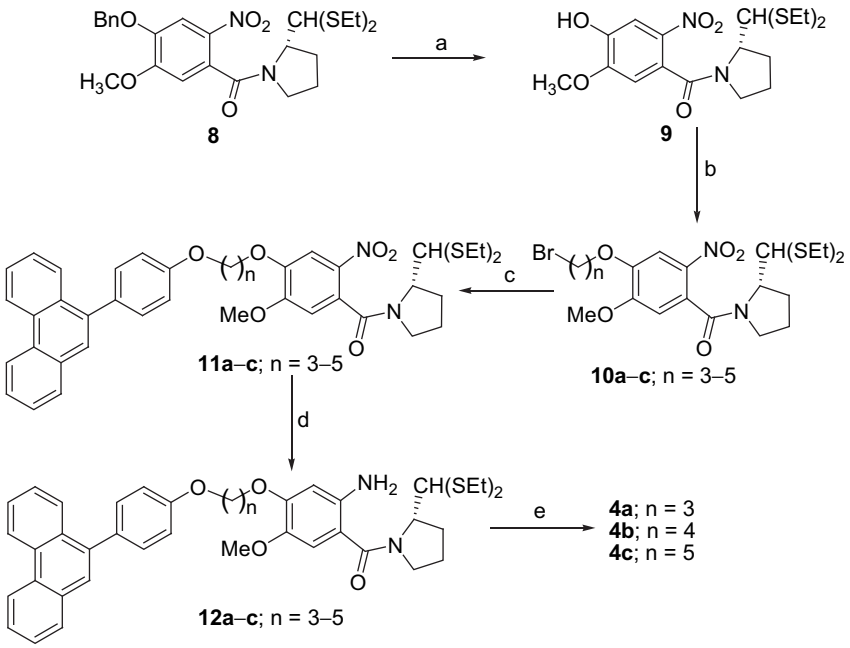
Representative compound **4a** has been evaluated by the National Cancer Institute to determine its growth inhibitory properties against 57 human cancer cell lines producing a growth inhibition (GI_{50}) ranging from 0.04 to 1.76 μM as shown in Table 1. Further, we examined the antiproliferative activity of these new PP-PBDs (**4a**–**c**) on human breast cancer cell line MCF-7 using MTT assay by treating cells at 0.5, 1, 2 and 4 μM of DC-81, compounds **4a**–**c** for 24 h.

The new PP-PBD conjugates exhibit enhanced potency over the parent compound DC-81 (Fig. 2). The results reveal that increasing the length of alkyl chain results in excellent cytotoxicity. Accordingly, the compound **4c** exhibited its activity to cause significant cytotoxicity (up to 50%) at 1 μM concentration while **4a** and **4b** show significant cytotoxicity (up to 50%) at 2 μM. Herein; the control cells have been taken as 100% in each of the compound treatment. All the compounds (**4a**–**c**) are equally effective at 4 μM. Thus, it can be concluded that compound **4c** is the most effective cytotoxic agent.

2.2.2. RED_{100}-restriction endonuclease digestion assay

Several reports in the literature have employed restriction endonuclease inhibition study to confirm the relative binding affinity of DNA interactive small molecule ligands [21–23]. A quantitative restriction enzyme digestion (RED_{100}) assay has been developed in which the inhibition of DNA cleavage by *Bam*HI is used to probe the DNA binding capability of PBD monomers [24]. We have earlier investigated this assay for preferences of base pair selectivity of imine–amide PBD dimers [25]. The drug when pre-incubated by PP-PBD compounds suggest that these molecules selectively interact with G-rich sequence of DNA. Therefore, PBD compounds with DNA were subjected to *Bam*HI digestion, and the protection of cleavage of the GGATCC sites is due to affinity of PBDs in covalent interaction with the free amino group attached to the N_2 of guanine in DNA.

In order to investigate the binding capability of these compounds to DNA at G-rich regions and inhibit restriction digestion by restriction endonuclease *Bam*HI, we have carried out the assay at various concentrations (2, 4, 8, 16 and 32 μM) of these compounds **4a**–**c**. Interestingly, the concentration dependent differential inhibitory activity by PBDs is observed for 100%



Scheme 2. *Reagents and conditions*: (a) $BF_3{\cdot}OEt_2$/EtSH, dry CH_2Cl_2, r.t., 8 h, 88%; (b) dibromoalkanes, K_2CO_3, dry DMF, r.t., 24 h, 88–93%; (c) **7**, K_2CO_3, dry acetone, reflux, 48 h, 85–90%; (d) $SnCl_2.2H_2O$, MeOH, reflux, 4 h, 75–80%; and (e) $HgCl_2$, $CaCO_3$, CH_3CN-H_2O (4:1), r.t., 12 h, 58–60%.

Table 1
GI_{50} values of compound **4a**[a] on 57 human cancer cell lines.

Cancer panel/cell line	GI_{50} (μM)	Cancer panel/cell line	GI_{50} (μM)
Leukemia		Melanoma	
CCRF-CEM	0.13	LOX IMVI	1.02
HL-60 (TB)	0.17	MALME-3M	1.59
K-562	0.27	M14	0.57
MOLT-4	0.18	SK-MEL-2	0.04
RPMI-8226	0.19	SK-MEL-28	1.26
SR	0.22	SK-MEL-5	1.06
		UACC-257	1.36
		UACC-62	0.63
Non-small cell lung cancer		Ovarian cancer	
A549/ATCC	0.72	IGROV1	0.12
EKVX	1.05	OVCAR-3	0.25
HOP-62	1.00	OVCAR-4	0.58
HOP-92	0.36	OVCAR-5	1.20
NCI-H226	1.17	OVCAR-8	0.42
NCI-H23	1.41	SK-OV-3	1.56
NCI-H322M	1.59		
NCI-H460	1.04	Prostate cancer	
NCI-H522	0.12	DU-145	0.40
Colon cancer		Renal cancer	
COLO 205	1.19	786-0	0.70
HCC-2998	1.21	A498	0.69
HCT-116	0.25	ACHN	1.26
HCT-15	1.18	CAKI-1	0.40
HT29	0.89	RXF 393	0.24
KM12	0.78	SN12C	0.51
SW-620	0.51	TK-10	0.60
		UO-31	0.16
CNS cancer		Breast cancer	
SF-268	0.86	MCF-7	0.17
SF-539	1.76	NCI/ADR-RES	1.67
SNB-19	1.16	MDA-MB-231/ATCC	0.29
SNB-75	0.21	HS-578T	1.02
U251	0.91	MDA-MB-435	0.24
		BT-549	0.27
		T-47D	0.08

[a] Data obtained from the NCI's in vitro disease-oriented human cancer cell line screening.

inhibition at 16 μM in all the PP-PBD compounds tested (Fig. 3). Based on the results of DNA binding studies and cytotoxicity, it has been considered of interest to understand the mechanism of tumour cell proliferation inhibition or cell death for these conjugates.

2.2.3. Cell cycle perturbations and apoptosis induction

Cell cycle analysis has been performed to explore the basis for antiproliferative properties of these PP-PBD conjugates (**4a–c**) at 2 μM and 4 μM concentrations in MCF-7 cells. G1 cell cycle arrest is observed in all the three compounds tested, as indicated by concentration dependent accumulation of cells in G0 phase. The control cells treated with DMSO (the solvent in which the compound is dissolved) showed 63.35% of G1 phase. Compound **4a** showed 73.46% and 83.68%, compound **4b** showed 81.39% and

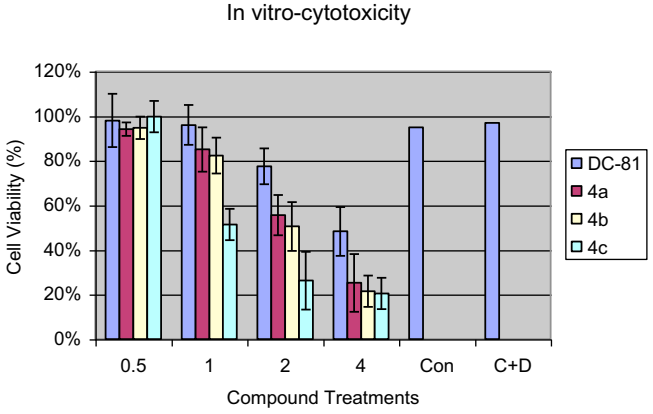


Fig. 2. Effect of PP-PBD compounds (**4a–c**) on cell viability (invitro cytotoxicity). MCF-7 cells were treated with 0.5, 1, 2 and 4 μM concentrations of PP-PBD compounds as indicated for 24 h in 96-well plates seeded with 10,000 cells/well. O.D. readings were taken at 570 nm wavelength to measure the percentage of cell viability after treatment with the respective compound. DC-81 was used as the positive control. Con: control cells (untreated cells), C + D: control cells treated with DMSO. Numbers 0.5, 1, 2 and 4 represent concentrations of the compounds in micromolar.

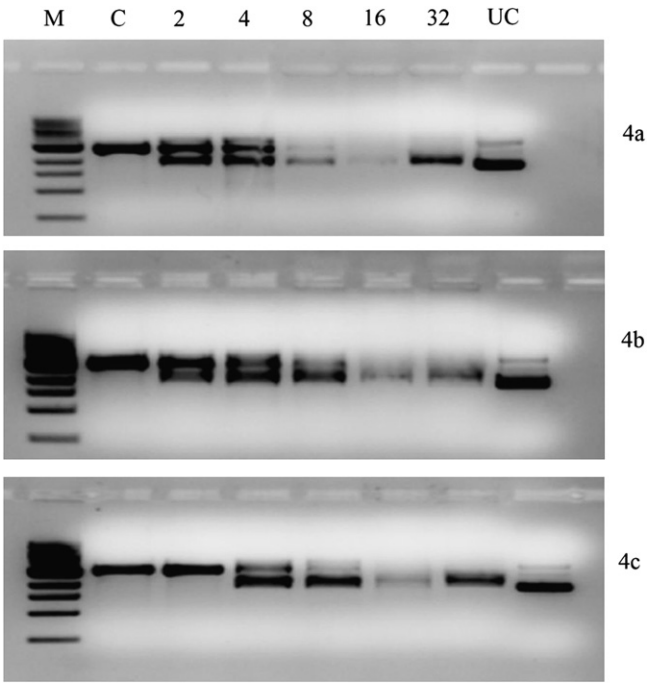


Fig. 3. Inhibitory activity of compounds **4a–c** on cleavage of PBR322 by restriction endonuclease *Bam*HI (10 U/μl) for 2 h at 37 °C. The cut (C) and the uncut (UC) were used as controls. 2, 4, 8, 16 And 32 represents concentrations in micromolar used for RED assay. Products were separated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination.

91.16% and compound **4c** showed 88.92% and 91.78% G1 phase at 2 μM and 4 μM concentrations, respectively. Whereas, DC-81 that has been taken as a positive control showed 69.17% and 83.15% of G1 phase. Therefore, compound **4c** could be considered as the most effective one to cause cell cycle arrest. Moreover, the increase of G0 and G1 phase and decrease of G2/M phase of cell cycle clearly indicates that these compounds follow an apoptotic pathway (Fig. 4a, b).

2.2.4. DNA fragmentation assay

The key feature of apoptotic response is the DNA ladder formation mediated by enzymes called caspase-3, 8 and 9, that are cysteine proteases [26]. DNA fragmentation assay has been carried out to investigate apoptosis by treating the MCF-7 cells with the PP-PBD conjugates. It is observed that DNA laddering is significant in compounds **4b** and **4c** at 4 μM concentration. The effectiveness of the compounds in causing DNA fragmentation is in the order of **4b** > **4c** > **4a** (Fig. 5). DNA laddering caused by the compounds depends on the p53 protein expression level. Earlier studies report that the threshold expression of p53 is important for DNA fragmentation [27]. It has been observed that p53 protein expression level is more in **4b** compared to **4c** (data has not shown). However, apart from p53, there could be some other proteins that may regulate this event.

2.2.5. Effect of PP-PBD conjugates on cyclin D1 levels

The cell cycle regulatory protein that control the G1 to S phase transition is cyclin D1 that is accumulated during late G1 phase of cell cycle [28]. In the present investigation the FACS data clearly indicates that cell cycle arrest occurs at G1 phase and possibly has an effect on cell cycle regulatory proteins particularly cyclin D1. The levels of cyclin D1 have been determined by western blot analysis for these PP-PBD conjugates (**4a–c)** in MCF-7 cells at 2 μM as well as 4 μM concentrations and compared to DC-81 (4 μM for 24 h). It is observed that the decrease in cyclin D1 is significant in case of compound **4c** thus indicating that the cells are effectively arrested at G1 phase of the cell cycle (Fig. 6).

2.2.6. PP-PBDs induced release of cytochrome c into the cytoplasm

Caspases are the family of cysteine proteases that are known to play a key role in the process of apoptosis [29]. Moreover, apoptosis occurs through two different pathways, that is the intrinsic and extrinsic pathways. Intrinsic pathway involves mitochondria that play an important role in apoptosis, whereas extrinsic pathway is mediated by cell death receptors such as Fas, TNF α after receiving the death signal. Cytochrome c levels in the cytoplasm have been determined by western blot analysis after treatment of MCF-7 cells with these conjugates (**4a–c)** at 2 μM as well as 4 μM, and compared to DC-81 (4 μM concentrations for 24 h). It is observed that there is an increase in cytosolic cytochrome c levels for all the compounds including for DC-81. These results clearly show that PP-PBD conjugates induce apoptosis through the intrinsic pathway involving mitochondria (Fig. 7).

2.2.7. Activation of caspase-8 and 9 by PP-PBD conjugates

As induction of apoptotic cell death is mediated by caspases, the involvement of various caspases and their role in the process of apoptosis has been investigated. MCF-7 cells lack endogenous caspase-3 protein that has been reported to play an important role in drug-induced apoptosis [29]. MCF-7 cells were treated with **4a–c** at 2 and 4 μM concentration and DC-81 at 4 μM. It is observed that caspase-8 activity is measured by Calorimetry method, increases when the MCF-7 cells were treated with PP-PBD conjugates (**4a–c**) in comparison to the untreated control cells and DC-81 (Fig. 8a).

Further it is determined that the caspase-9 activity increases significantly when MCF-7 cells have been treated with **4c** at 2 μM and 4 μM concentrations using a fluorimetry based assays. This activity reduced to the levels below that of control when caspase-9 inhibitor (LEHD-CHO) is added. A similar increase of caspase-9 activity (by twofolds) in comparison to untreated controls has been observed when cells were treated with compounds **4a** and **4b** at 4 μM concentrations (Fig. 8b).

2.2.8. Induced cleavage of PARP by PP-PBD conjugates

Activation of caspases during apoptosis results in the cleavage of PARP (Poly ADP-ribose Polymerase) a 116 kDa protein [30]. Cleavage of PARP inactivates the enzyme and hence cannot bind to DNA strand breaks during drug-induced apoptosis in many different cell lines [31]. Since these compounds have shown an increase in the caspase-9 activity and cytochrome c release, it is considered of interest to understand their effect on cleavage of PARP by western blot analysis using antiPARP antibody (89 kDa) on MCF-7 cells. It is observed that there is an increase of cleaved PARP expression in all the compounds tested as shown in Fig. 7.

3. Conclusion

We have synthesized a set of phenanthrylphenol-PBD conjugates and evaluated them for the anticancer properties by a battery of assays. RED_{100} assay has revealed that these compounds show effective DNA binding activity. MTT assay, which indicates cytotoxicity, has revealed that PP-PBD derivative **4c** to be highly effective. Flow cytometric data of these compounds showed increased sub G1 peak, which is suggestive of apoptosis and G1 cell cycle arrest. It is known that over expression of cyclin D1 increases the phosphorylation status of retinoblastoma protein and transition from G1 to S phase. Furthermore, MCF-7 cells have been found to be dependent on cyclin D1/CDK4 pathway for their continued proliferation. Interestingly, these compounds are effective as cyclin D1

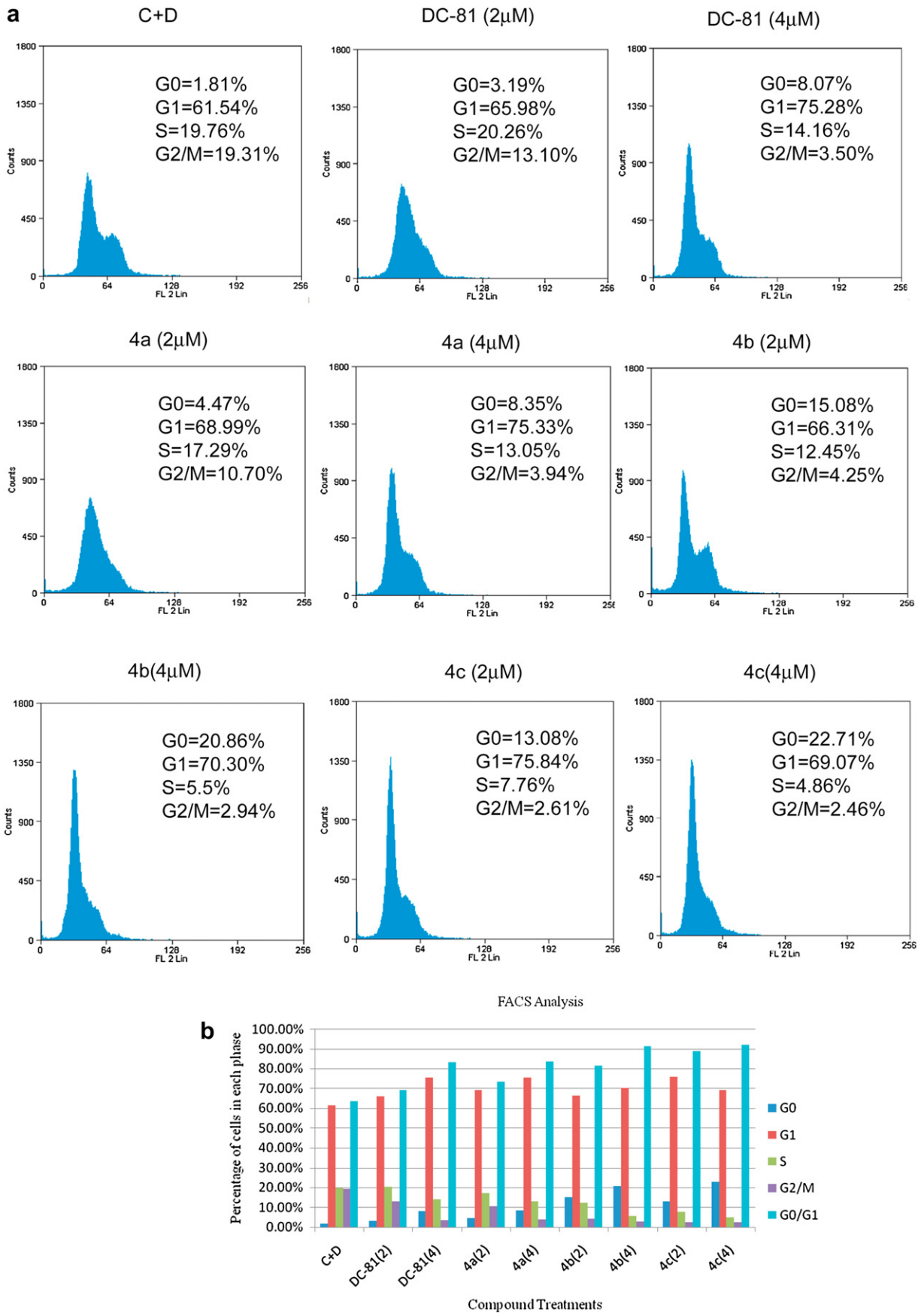


Fig. 4. (a) DNA histograms obtained by flowcytometry. The percentages of cells in sub G0, G1, S, and G2/M cell cycle phase, after the treatment of MCF-7 cells with **4a–c** at 2 μM and 4 μM for 24 h. (b). FACS analysis of cell cycle distribution of MCF-7 cells after treatment with PP-PBD conjugates (**4a–c**) for 24 h. DC-81 was used as the positive control. C + D: control cells treated with DMSO. Numbers 2 and 4 in parenthesis represent concentrations of the treated compounds in micromolar.

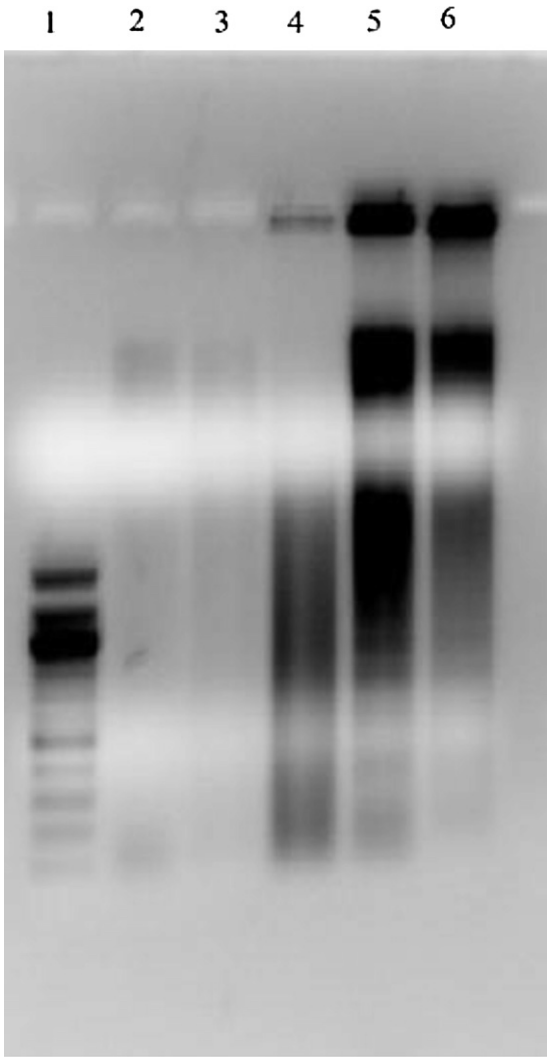


Fig. 5. Agarose gel electrophoresis of DNA extracted from MCF-7 cells. The figure represents MCF-7 cells treated with PP-PBD compounds for 24 h. DNA from the cells was extracted and electrophoresed in 1% agarose gel and visualized by ethidium bromide staining under UV illumination. Lane 1: 100 bp marker, Lane 2: control cells (without treatment), Lane 3: control cells + DMSO, Lane 4: treated by PP-PBD (**4a**-4 μM). Lane 5: treated with PP-PBD (**4b**-4 μM). Lane 6: treated with PP-PBD (**4c**-4 μM). Lanes 5 and 6 show a clear DNA fragmentation.

inhibitors and have shown a pronounced increase in caspase-9 activity, cytosolic cytochrome c and cleaved PARP. Moreover, a correlation has been obtained with the earlier studies that have demonstrated that cyclin D1 regulates the size and function of mitochondria. Therefore, these results support the inhibition of cyclin D1/CDK4 complex and development of such inhibitors as potential anticancer agents.

4. Experimental protocols

Reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 and 60–120 mesh). The majority of reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenone ketyl), methanol (magnesium methoxide), and acetonitrile (calcium

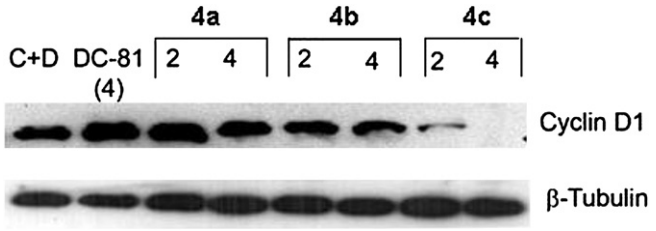


Fig. 6. Effect of PP-PBD compounds on cyclin D1 levels. MCF-7 cells were treated with PP-PBDs **4a–c** at 2 μM, 4 μM and DC-81 at 4 μM concentrations for 24 h. The cell lysates were collected and expression levels of cyclin D1 were determined by western blot analysis. β-Tubulin was used as loading control. C + D: control + DMSO.

hydride). ^{1}H NMR spectra were recorded on Varian Gemini 200 MHz and Avance 300 MHz spectrometer using tetramethyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) down field from tetramethyl silane. Spin multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Coupling constants are reported in Hertz (Hz). Optical rotations are measured on Horiba, high sensitive polarimeter, SEPA-300.

4.1. Synthesis of phenanthrylphenol-PBD conjugates

4.1.1. 9-(4-Methoxyphenyl) phenanthrene (**6**)

To a stirred solution of 9-bromophenanthrene (257 mg, 1.0 mmol) in dioxane/H_2O (10 mL/2 mL) in a sealable tube, was added 4-methoxyphenylboronic acid (**5**) (304 mg, 2.0 mmol) and K_3PO_4 (636 mg, 3.0 mmol). The mixture was bubbled with N_2 over 5 min, and then $Pd(PPh_3)_4$ (110 mg, 0.1 mmol) was added and heated to 80 °C. The reaction mixture was then poured into water (10 mL) and extracted with ethyl acetate (4 × 15 mL). The combined organic phases were washed with H_2O, brine and dried over Na_2SO_4. After removal of solvent, the residue was purified by column chromatography. (198 mg, 70%): $R_f = 0.85$ (EtOAc–hexane 1:9); White solid, mp: 142–144 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 3.90 (s, 3H, $-OC\mathbf{H_3}$), 7.0 (d, $J = 7.8$ Hz, 2H, Ar**H**), 7.4 (d, $J = 7.8$ Hz, 2H, Ar**H**), 7.48–7.73 (m, 5H, ArH), 7.79–7.99 (m, 2H, Ar**H**), 8.70 (m, 2H, Ar**H**).

4.1.2. 4-(9-Phenanthryl)phenol (**7**)

Compound **6** (284 mg, 1.0 mmol) was dissolved in dry CH_2Cl_2 (5 mL) to which borontribromide (35 mmol) was slowly added. This solution was stirred at −78 °C for 1 h under nitrogen atmosphere. After this procedure, the reaction was slowly brought to ambient temperature. TLC was indicated that the reaction reached by completion usually within 5 h. Ice was added slowly to this mixture and was neutralized with aqueous ammonium chloride and then

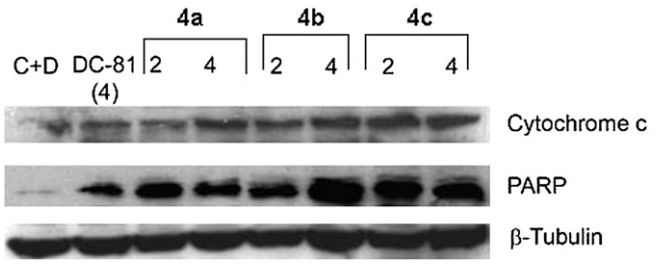


Fig. 7. Effect of PP-PBD compounds on cytochrome c release and levels of cleaved PARP. MCF-7 cells were treated with compounds **4a–c** at 2 μM, 4 μM and DC-81 at 4 μM concentrations for 24 h. The cell lysates were collected and expression levels of cytochrome c and cleaved PARP were determined by western blot analysis. β-Tubulin was used as loading control. C + D: control + DMSO.

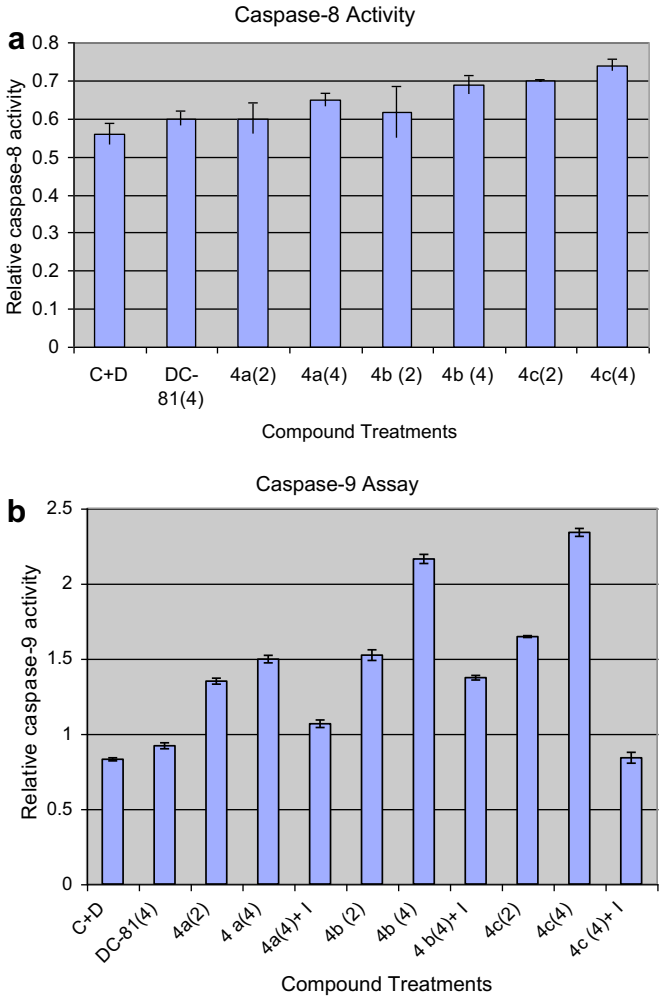


Fig. 8. (a) Effect of PP-PBD conjugates on caspase-8 activity in MCF-7 cells. The increased enzymatic activity of caspase-8, in apoptosis after the treatment of PP-PBDs (**4a–c**) was determined by photometry. The cleavage of peptide by caspase-8 releases the pNA, which can be quantified at wavelength of 405 nm using multimode varioskan Flash. The concentrations of the compounds in micromolar (μM) are represented in brackets. Here DC-81 was used as positive control. Numbers 2 and 4 in brackets represent concentrations of the treated compounds in micromolar. (b). Effect of PP-PBD conjugates on caspase-9 activity in MCF-7 cells. The increased enzymatic activity of caspase-9, in apoptosis after the treatment of PP-PBDs (**4a–c**) was determined by flourimetry. The cleavage of peptide by caspase-9 releases the fluorophore AMC that was quantified at excitation wavelength of 400 nm and emission wavelength of 505 nm. The concentrations of the compounds in micromolar (μM) are represented in brackets. 'I' represents the inhibitor used. DC-81 was used as the positive control. C + D: control cells treated with DMSO. Numbers 2 and 4 in brackets represent concentrations of the treated compounds in micromolar.

organic layer was washed with water (3 × 10 mL). After drying by Na_2SO_4, the product was concentrated under reduced pressure. The target product was isolated using column chromatography. (216 mg, 80%): $R_f = 0.79$ (EtOAc–hexane 1:9); White solid, mp: 150–152 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 6.93 (d, $J = 8.59$ Hz, 2H, Ar**H**), 7.4 (d, $J = 8.59$ Hz, 2H, Ar**H**), 7.46–7.71 (m, 5H, Ar**H**), 7.80–7.97 (m, 2H, Ar**H**), 8.71 (m, 2H, Ar**H**).

*4.1.3. (2S)-N-{4-(3-[4-(9-Phenanthryl)phenoxy]propyl)oxy-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehyde diethyl thioacetal (**11a**)*

To a solution of (2*S*)-*N*-[4-(3-bromopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**10a**) (521 mg, 1 mmol) in acetone (10 mL) was added anhydrous K_2CO_3 (552 mg, 4 mmol) and 4-(9-phenanthryl)phenol (**7**) (270 mg, 1 mmol). The reaction mixture was heated to reflux for 48 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product thus obtained was purified by column chromatography to afford pure compound **11a** (639 mg, 90%); $R_f = 0.61$ (EtOAc–hexane 3:7); Light yellow solid, mp: 64–66 °C; $[\alpha]_D^{23} = -58^\circ$ ($c = 0.9$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.16–1.45 (m, 6H, −(C**H**$_3$)$_2$), 1.51–1.91 (m, 2H, −C**H**$_2$−) 2.26–2.35 (m, 2H, −C**H**$_2$CH$_2$−), 2.50–2.62 (m, 2H, −C**H**$_2$−), 2.63–2.90 (m, 4H, 2×−SCH$_2$−), 3.16–3.32 (m, 2H, −NC**H**$_2$−), 3.99 (s, 3H, −OC**H**$_3$), 4.27 (t, $J = 5.7$ Hz, 2H, −OC**H**$_2$-), 4.36 (t, $J = 5.7$ Hz, 2H, −OC**H**$_2$−), 4.65–4.72 (m, 1H, −NC**H**−), 4.8 (d, $J = 3.0$ Hz, 1H, −C**H**S$_2$−), 6.82 (s, 1H, Ar**H**), 7.06 (d, $J = 8.6$ Hz, 2H, ArH), 7.45 (d, $J = 8.6$ Hz, 2H, ArH), 7.50–7.72 (m, 5H, Ar**H**), 7.76 (s, 1H, Ar**H**), 7.82–8.07 (m, 2H, ArH), 8.63–8.82 (m, 2H, Ar**H**). MS (ESI): *m/z* 711 $[M + H]^+$.

*4.1.4. (2S)-N-{4-(4-[4-(9-Phenanthryl)phenoxy]butyl)oxy-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehyde diethyl thioacetal (**11b**)*

The compound **11b** was prepared according to the method described for the compound **11a** by employing compounds **7** and **10b** (535 mg, 1 mmol) to afford the crude compound, which was further purified by column chromatography affords compound **11b** (638 mg, 88%); Light yellow solid, $R_f = 0.62$ (EtOAc–hexane 3:7); mp: 60–62 °C; $[\alpha]_D^{23} = -66^\circ$ ($c = 0.86$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.16–1.42 (m, 6H, −(C**H**$_3$)$_2$), 1.75–1.93 (m, 4H, 2×−C**H**$_2$−), 2.05–2.32 (m, 4H, 2×−C**H**$_2$−), 2.67–2.89 (m, 4H, 2×−SC**H**$_2$−), 3.15–3.30 (m, 2H, −NC**H**$_2$−), 3.89 (s, 3H, −OC**H**$_3$), 4.03–4.31 (m, 4H, −OC**H**$_2$−), 4.63–4.77 (m, 1H, −NC**H**−), 4.87 (d, $J = 3.6$ Hz, 1H, −C**H**S$_2$−), 6.82 (s, 1H, Ar**H**), 7.03 (d, $J = 8.6$ Hz, 2H, Ar**H**), 7.46 (d, $J = 7.9$ Hz, 2H, Ar**H**), 7.51–7.74 (m, 6H, Ar**H**), 7.83–8.05 (m, 2H, Ar**H**), 8.66–8.81 (m, 2H, Ar**H**); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 166.5, 154.4, 138.3, 137.2, 133.1, 131.4, 131.1, 130.6, 129.7, 128.5, 128.1, 127.3, 126.7, 126.3, 122.8, 122.4, 114.2, 109.2, 108.1, 69.2, 67.3, 61.0, 56.4, 52.8, 50.1, 27.2, 26.6, 26.2, 26.0, 25.7, 24.6, 15.1, 14.9 ppm; MS (ESI): *m/z* 725 $[M + H]^+$; Anal. calcd for $C_{41}H_{44}N_2O_6S_2$: C 67.93, H 6.12, N 3.86, S 8.85, found: C 67.72, H 6.48, N 3.58, S 8.44.

*4.1.5. (2S)-N-{4-(5-[4-(9-Phenanthryl)phenoxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehydediethyl thioacetal (**11c**)*

The compound **11c** was prepared according to the method described for the compound **11a** by employing compounds **7** and **10c** (457 mg, 1 mmol) to afford the crude compound, which was purified by column chromatography, affords compound **11c**. (628 mg, 85%); $R_f = 0.63$ (EtOAc–hexane 3:7); Light yellow solid, mp: 58–60 °C; $[\alpha]_D^{23} = -54^\circ$ ($c = 1$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.19–1.42 (m, 8H, −(C**H**$_3$)$_2$, 2×−C**H**$_2$−), 1.44–1.62 (m, 2H, −C**H**$_2$−), 1.68–1.87 (m, 2H, −C**H**$_2$−), 1.90–2.05 (m, 4H, −C**H**$_2$−), 2.62–2.88 (m, 4H, 2×−SC**H**$_2$−), 3.14–3.33 (m, 2H, −C**H**$_2$−), 3.89 (s, 3H, −OC**H**$_3$), 4.06–4.20 (m, 4H, 2×−OC**H**$_2$−), 4.63–4.78 (m, 1H, −C**H**S$_2$−), 4.86 (d, $J = 3.7$ Hz, 1H, −NC**H**−), 6.80 (s, 1H, Ar**H**), 7.01 (d, $J = 9.0$ Hz, 2H), 7.44 (d, $J = 8.3$ Hz, 2H, Ar**H**), 7.47–7.71 (m, 6H, Ar**H**), 7.83–7.95 (m, 2H, Ar**H**), 8.66–8.79 (m, 2H, Ar**H**); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 166.6, 158.4, 154.4, 148.4, 138.3, 137.2, 133.0, 131.5, 131.0, 130.6, 129.7, 128.5, 128.0, 127.3, 126.7, 126.3, 122.8, 122.4, 114.2, 109.2, 108.1, 69.4, 67.6, 61.0, 56.4, 52.8, 50.1, 28.9, 28.6, 27.1, 26.6, 26.2, 24.6, 22.6, 15.1, 14.9 ppm; MS (ESI): *m/z* 739 $[M + H]^+$; Anal. calcd. for $C_{42}H_{46}N_2O_6S_2$: C 68.27, H 6.27, N 3.79, S 8.68, found: C 67.99, H 6.22, N 3.46, S 8.42.

*4.1.6. 7-Methoxy-8-[N-(4-(9-phenanthryl)phenoxy)propyl]oxy-(11aS)-1,2,3,11a-tetrahy-dro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one (**4a**)*

A solution of amino thioacetal **12a** (681 mg, 1 mmol), $HgCl_2$ (596 mg, 2.2 mmol), and $CaCO_3$ (240 mg, 2.4 mmol) in CH_3CN–H_2O

(16:4) was stirred slowly at room temperature until TLC (EtOAc) indicates complete loss of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through celite. The clear yellow organic supernatant was extracted with ethyl acetate (2 × 20 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (2 × 20 mL), brine (2 × 20 mL), and then combined organic phase was dried (Na_2SO_4). The organic layer was evaporated under vacuum and the crude product was purified by column chromatography to afford the compound **4a** as a white solid; (334 mg, 60%); $R_f = 0.70$ (MeOH–$CHCl_3$, 1:9); mp 112–114 °C. $[\alpha]_D^{23} = +448^{\circ}$ ($c = 1$ in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.02–2.13 (m, 2H, −**CH$_2$**−), 2.27–2.38 (m, 2H, −**CH$_2$**−), 2.39–2.51 (p, 2H, −**CH$_2$**−), 3.54–3.66 (m, 1H, −N**CH$_2$**−), 3.69–3.78 (m, 1H, −N**CH$_2$**−), 3.79–3.91 (m, 1H, −N**CH**−), 3.98 (s, 3H, −O**CH$_3$**), 4.28–4.38 (m, 4H, 2×−O**CH$_2$**−), 6.92 (s, 1H, Ar**H**), 7.08 (d, $J = 9.0$ Hz, 2H, Ar**H**), 7.47 (d, $J = 9.0$ Hz, 2H, Ar**H**), 7.56 (s, 1H, Ar**H**), 7.58–7.72 (m, 6H, 5×Ar**H,** imine-**H**), 7.88–7.99 (m, 2H, Ar**H**), 8.71–8.83 (m, 2H, Ar**H**); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 161.6, 136.3, 131.1, 127.1, 123.5, 121.3, 119.7, 111.8, 102.8, 61.6, 39.2, 31.9, 28.9, 22.9 ppm; MS (ESI): *m/z* 557 $[M + H]^+$; HRMS-EI: *m/z* $[M + Na]^+$ calcd for $C_{36}H_{32}N_2O_4$ 579.2259, found 579.2233; Anal. calcd for $C_{36}H_{32}N_2O_4$: C 77.68, H 5.79, N 5.03 found: C 77.26, H 5.44, N 4.87.

4.1.7. 7-Methoxy-8-[N-(4-(9-phenanthryl)phenoxy)butyl]oxy-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one (**4b**)

The compound **4b** has been prepared by following the method described in the compound **4a** employing 12b (695 mg, 1 mmol) to afford the compound **4b** as a white solid (342 mg, 60%); $R_f = 0.72$ (MeOH–$CHCl_3$, 1:9); mp 102–104 °C; $[\alpha]_D^{23} = +250^{\circ}$ ($c = 0.9$ in $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.95–2.23 (m, 6H, 3×−C**H$_2$**−), 2.25–2.40 (m, 2H, −C**H$_2$**−), 3.52–3.67 (m, 1H, −N**CH$_2$**−), 3.64–3.77 (m, 1H, −N**CH$_2$**−), 3.78–3.90 (m, 1H, −N**CH**−) 3.96 (s, 3H, −OC**H$_3$**), 4.10–4.26 (m, 4H, 2×−OC**H$_2$**−), 6.85 (s, 1H, Ar**H**), 7.05 (d, $J = 9.0$ Hz, 2H, Ar**H**), 7.46 (d, $J = 8.3$ Hz, 2H, Ar**H**), 7.53 (s, 1H, ArH), 7.55–7.70 (m, 6H, 5×Ar**H**, imine-**H**), 7.86–7.98 (m, 2H, Ar**H**), 8.70–8.80 (m, 2H, Ar**H**); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 162.2, 158.2, 150.6, 147.6, 140.4, 138.2, 132.8, 131.3, 130.9, 130.4, 129.6, 128.4, 127.2, 126.7, 126.2, 122.5, 120.0, 114.1, 111.3, 110.2, 68.5, 67.3, 56.1, 53.6, 46.7, 29.6, 25.9, 24.2 ppm; MS (ESI): *m/z* 571 $[M + H]^+$; Anal. calcd for $C_{37}H_{34}N_2O_4$: C 77.87, H 6.01, N 4.91 found: C 77.59, H 6.00, N 4.37.

4.1.8. 7-Methoxy-8-[N-(4-(9-phenanthryl)phenoxy)pentyl]oxy-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one (**4c**)

The compound **4c** has been prepared following the method described in the compound **4a** employing **12c** (709 mg, 1 mmol) to afford the compound **4c** as a white solid (339 mg, 58%); $R_f = 0.75$ (MeOH–$CHCl_3$, 1:9); mp 96–98 °C; $[\alpha]_D^{23} = +384^{\circ}$ ($c = 0.89$ in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.62–1.83 (m, 2H, −**CH$_2$**−), 1.85–2.16 (m, 4H, −**CH$_2$**−), 2.23–2.45 (m, 2H, −C**H$_2$**−), 3.38 (t, $J = 6.7$, 7.5 Hz, 2H, −C**H$_2$**−), 3.51–3.64 (m, 1H, −N**CH$_2$**−), 3.67–3.76 (m, 1H, −N**CH$_2$**−), 3.78–3.89 (m, 1H, −N**CH**−), 3.96 (s, 3H, −O**CH$_3$**), 3.99–4.27 (m, 4H, 2×−O**CH$_2$**−), 6.85 (s, 1H, Ar**H**), 7.05 (d, $J = 7.8$ Hz, 2H, Ar**H**), 7.47 (d, $J = 8.5$ Hz, 2H, Ar**H**), 7.54 (s, 1H, Ar**H**), 7.63–7.75 (m, 6H, 5×Ar**H,** imine-**H**), 7.85–8.01 (m, 2H, Ar**H**), 8.68–8.83 (m, 2H, Ar**H**); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 164.4, 162.2, 158.2, 150.6, 140.6, 140.4, 138.3, 132.7, 131.5, 131.1, 130.9, 130.5, 129.6, 128.4, 127.3, 126.7, 126.2, 122.7 122.4, 120.0, 114.2, 111.4, 110.5, 68.8, 67.6, 56.2, 53.7, 46.6, 29.6, 29.1, 28.7, 24.2, 22.7 ppm; MS (ESI): *m/z 585* $[M + H]^+$; Anal. calcd for $C_{38}H_{36}N_2O_4$: C 78.06, H 6.21, N 4.79, found: C 77.84, H 6.13, N 4.27.

4.2. Cell lines

MCF-7 (human breast cancer cells) was obtained from ATCC, USA. MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum and 100 U/mL Pencillin and 100 μg/mL streptomycin sulfate (Sigma). The cells were passaged and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

4.3. Cell viability (MTT assay)

Cell viability was assessed by the MTT assay using Vybrant MTT cell proliferation assay kit (Invitrogen), which is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. Briefly, MCF-7 cells were seeded in a 96-well plate (TPP) at a cell density of 10,000 cells/well. After overnight incubation, the cells were treated with compounds **4a–c** and DC-81, and incubated for 24 h. The medium was then discarded and replaced with fresh 100 μL media followed by addition of 10 μL of MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubulized in 100 μL of extraction buffer (SDS). The optical density (O.D.) was read at 570 nm using Multimode Varioskan FLASH (Thermoscientifics).

4.4. Cell cycle analysis

5×10^5 Cells of MCF-7 were seeded in 60 mm dish and were allowed to grow for 24 h. Compounds **4a–c** and DC-81 at 2 and 4 μM were added to the culture media and the cells were incubated for an additional 24 h. Harvesting of cells was done with Trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, washed with PBS and incubated with 1 mg/mL RNaseA solution (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm for 5 min and further stained with 250 μl of DNA staining solution [10 mg of Propidium Iodide (PI), 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of sterile MilliQ water at room temperature for 30 min in the dark]. The DNA contents of 20,000 events were measured by flowcytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

4.5. RED_{100} assay

The restriction endonuclease digestion is based on the activity of a compound to inhibit the cleavage activity of restriction endonuclease. Compounds **4a–c** with the final concentrations of 2, 4, 8, 16 and 32 μM were added to the PBR322 vector DNA and incubated for 16 h at 37 °C and restriction enzyme (*Bam*HI) was added to check whether the compound binds to DNA and inhibits the enzymatic digestion. The digestion assay gel includes two controls uncut (UC) PBR322 DNA and C indicates cut (without the addition of the compound). The plasmid (1 μg) was incubated with each of the above compound in a final volume of 17 μl for 16 h at 37 °C. Next 10× *Bam*HI buffer (2 μl) and 1 μl of 10 U/μl of *Bam*HI enzyme was added and incubated for 2 h at 37 °C. The contents were loaded on to 1% agarose gel. The electrophoresis was carried out in 0.5 × Tris–acetate EDTA buffer at 80 V for 1 h and the gel was stained with ethidium bromide and photographed.

4.6. DNA fragmentation assay

The 1.5×10^6 MCF-7 cells were grown in 60 mm dish and treated with compounds **4a–c** at a concentration of at 4 μM, the effective concentration which was obtained from FACS analysis. The cells were washed twice in 1× TBS and lysed in 10 mM Tris–Hcl pH 7.5; 10 mM EDTA and 0.5% Triton X-100. The lysate was then centrifuged at 13 K for 10 min and the supernatant was treated with 1 μg of RNaseA and 40 μg of proteinase K for 2 h at 37 °C, respectively. After phenol–chloroform extraction, the DNA was precipitated with

1/10th volume of 3 M sodium acetate and 2 volumes of ethanol and stored at −70 °C for 2 h and centrifuged at 12 K for 30 min. After 70% ethanol wash the DNA samples were analyzed by agarose gel electrophoresis in 1.2% agarose gel containing 1 μl of 10 mg/mL ethidium bromide.

4.7. Caspase-8 assay

Caspase-8 assay was carried out using the Apoalert caspase-8 calorimetry assay kit (Clonetech, CA) according to manufacturer's recommendations. MCF-7 cells were treated with compounds **4a–c** and DC-81 and the substrate used in this assay was IETD-pNA. The caspase-8 substrate and cell lysate was incubated at 37 °C for 1 h and readings were taken at 405 nm.

4.8. Caspase-9 assay

We have used Apoalert caspase-9/6 fluorescent assay kit (Clonetech, CA) according to the manufacturer's recommendations. MCF-7 cells were treated with compounds **4a–c** and DC-81 at 2 and 4 μM concentrations as obtained from FACS analysis. Here the substrate and inhibitor (I) used are LEHD-AMC and LEHD-CHO, respectively. The LEHD-AMC substrate, LEHD-AMC + LEHD-CHO is added to the cell lysate and incubation was carried out at 37 °C for 1 h. Readings were taken at excitation wavelength 400 nm and emission wavelength 505 nm.

4.9. Protein extraction and western blot analysis

Total cell lysates from cultured MCF-7 cells after compound treatments as mentioned earlier were obtained by lysing the cells in ice-cold RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and containing 100 μg/mL PMSF, 5 μg/mL Aprotinin, 5 μg/mL Leupeptin, 5 μg/mL Pepstatin and 100 μg/mL NaF. After centrifugation at 12,000 rpm for 10 min, the protein in supernatant was quantified by Bradford method (BIO-RAD) using Multimode varioscan instrument (Thermo Fischer Scientifics). Thirty micrograms of protein per lane was applied in 12% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in TBS + 0.1% Tween20 (TBST) containing 5% blocking powder (Santacruz). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight (O/N). Rabbit polyclonal β-Tubulin (1:100), mouse monoclonal cytochrome c (1:300), rabbit polyclonal cyclin D1 (1:200) and mouse monoclonal PARP (1:200) antibodies were purchased from Imgenex, USA. After three TBST washes, the membrane was incubated with corresponding horseradish peroxidase-labeled secondary antibody (1:2000) (Santa Cruz) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min and the protein blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd.). The X-ray films were developed with developer and fixed with fixer solution.

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