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Design and synthesis of benzo[*c,d*]indolone-pyrrolobenzodiazepine conjugates as potential anticancer agents

Ahmed Kamal [a,c,*], G. Ramakrishna [a], V. Lakshma Nayak [b], P. Raju [a], A.V. Subba Rao [a], A. Viswanath [a], M.V.P.S. Vishnuvardhan [b], Sistla Ramakrishna [b,*], G. Srinivas [d]

[a] Division of Organic Chemistry, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500 607, India
[b] Division of Pharmacology, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500 607, India
[c] Catalytic Chemistry Chair, Chemistry Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[d] Centre for Cellular and Molecular Biology, Tarnaka, Hyderabad, 500 607, India

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ABSTRACT

A series of benzo[*c,d*]indol-2(1*H*)one-PBD conjugates (**11a–l**) have been designed and synthesized as potential anticancer agents. These compounds were prepared by linking the C8-position of DC-81 with a benzo[*c,d*]indol-2(1*H*)one moiety through different alkane spacers in good yields and confirmed by ^{1}H NMR, mass and HRMS data. The DNA binding ability of these conjugates was evaluated by thermal denaturation studies and interestingly, compound **11l** showed enhanced DNA binding ability. These compounds were also evaluated for their anticancer activity in selected human cancer cell lines of lung, skin, colon and prostate by using MTT assay method. These new conjugates showed promising anticancer activity with IC_{50} values ranging from 1.05 to 36.49 μM. Moreover, cell cycle arrest in SubG1 phase was observed upon treatment of A549 cells with 1 and 2 μM (IC_{50}) concentrations of compound **11l** and it induced apoptosis. This is confirmed by Annexin V-FITC, Hoechst staining, caspase-3 activity as well as DNA fragmentation analysis.

1. Introduction

With more than 10 million new cases every year, cancer remains one of the world's most devastating diseases. From medicinal chemistry point of view DNA has become an important target for cancer chemotherapy. Generally, 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.[1–3] Anticancer activity of DNA intercalative drugs is due to their insertion between the base pairs of the double helix and by forming hydrogen bonds with them. DNA intercalators have been developed for more than 40 years.[4] Some of the successful candidates are amonafide,[5] mitonafide,[6] crisnatol (770U82)[7–10] and all these compounds are in clinical trials at different stages. Previously, benzo[*c,d*]indol-2(1*H*)one derivatives were synthesized and developed as dyes to prepare electronic typing materials. Recently, Quin and coworkers reported benzo[*c,d*]indol-2(1*H*)one derivatives as novel DNA intercalators without basic side chains and as efficient antitumour agents.[11]

Pyrrolobenzodiazepines are a group of potent, naturally occurring anti-tumour antibiotics produced by various *Streptomyces* species which includes anthramycin, tomaymycin, sibiromycin, neothramycins A, B and DC-81 (Fig. 1). These compounds showed anticancer activity due to their covalent binding to the C2–NH_2 group of a guanine base through the electrophillic C11-position of the PBD in the minor groove of DNA.[12] PBDs are also known to bind sequence-selectively with DNA and target 5-Pu-G-Pu sequences.[13,14] Because of their natural right-handed twist due to the *S*-configuration at C11a position, these compounds strongly fit into the minor groove of DNA. The previous structure–activity relationship (SAR) studies have established that the linkage at C8-position with another DNA intercalator could greatly influence the biological activity. Previously we have reported PBD conjugates that are linked to a DNA intercalating moiety and examined their DNA binding ability as well as anticancer activity.[15–18] These studies demonstrated that such PBD conjugates exhibit enhanced DNA binding ability and anticancer activity compared to their individual scaffolds. Interestingly, pyrene-linked PBD hybrids have shown promising anticancer activity and are presently undergoing preclinical studies.[19]

* Corresponding authors. Tel.: +91 40 27193157; fax: +91 40 27193189.
E-mail addresses: ahmedkamal@iict.res.in (A. Kamal), sistla@iict.res.in (S. Ramakrishna).

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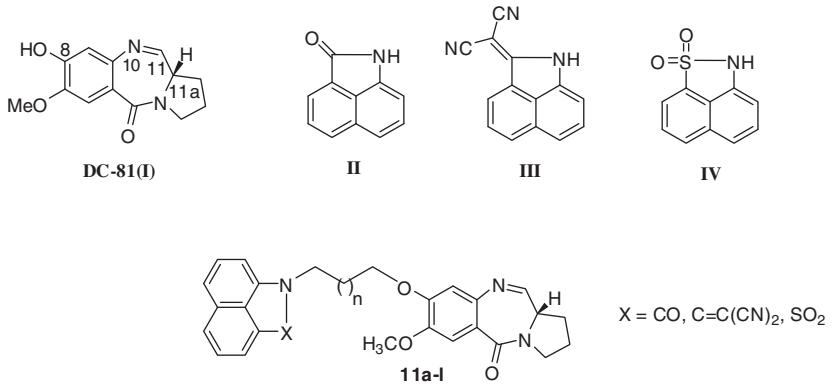


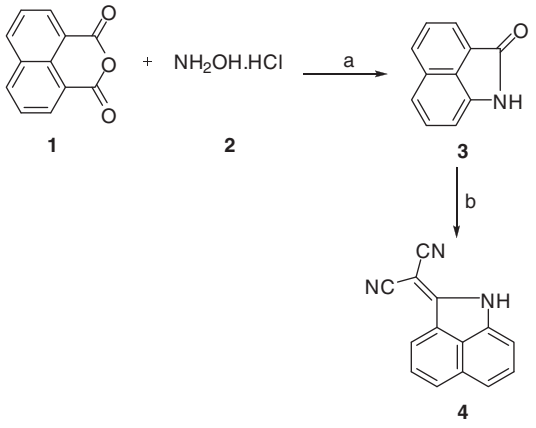
Figure 1. Structures of DC-81 (**I**), benzo[*c,d*]indol-2(1*H*)one derivatives (**II, III, IV**) and benzo[*c,d*]indol-2(1*H*)one-PBD conjugates (**11a–l**).

In continuation of these efforts in search of new anticancer agents, we have synthesized benzo[*c,d*]indol-2(1*H*)one-PBD conjugates and evaluated their anticancer potential. The significant DNA binding ability and prominent cytotoxicity of these conjugates encouraged us to evaluate detailed mechanistic pathway. The FACS data of the conjugate **11l** showed the cell cycle arrest at sub G1 stage.

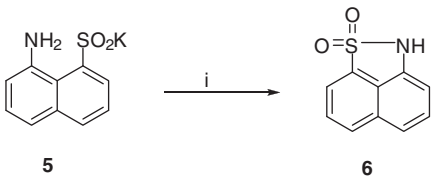
2. Chemistry

Synthesis of indolone intermediates **3, 4** and **6** was carried out from the compounds **1** and **5**. Compound **1** on reaction with hydroxyl amine hydrochloride in dry pyridine gave compound **3**. Benzo[*c,d*]indol-2(1*H*)-one (**3**) was reacted with propanedinitrile in toluene at 100 °C in presence of $POCl_3$ for 4 h to give compound **4** in quantitative yield. Further, cyclization of compound **5** with $POCl_3$ at 130 °C gave compound **6** as shown in Schemes 1 and 2.

Etherification of compound **7** with dibromoalkanes in the presence of potassium carbonate gave compounds **8a–d**. Synthesis of C8-linked benzo[*c,d*]indol-2(1*H*)-one-PBD conjugates (**11a–l**) was carried out from the (2*S*)-*N*-{4-[3-bromoalkoxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**8a–d**), and these were prepared by employing the previously reported methods from this laboratory.[20–25] These upon etherification with benzo[*c,d*]indol-2(1*H*)-one (**3, 4** and **6**) intermediates using sodium hydride in dry DMF provided corresponding nitro thioacetals (**9a–l**). These nitro thioacetals **9a–l** were reduced to the amino thioacetals **10a–l** by employing $SnCl_2{\cdot}2H_2O$ in refluxing MeOH and then cyclized by treatment with $HgCl_2$ and $CaCO_3$ in MeCN–H_2O to yield the target products **11a–l** as shown in Scheme 3.



Scheme 1. Reagents and conditions: (a) Dry pyridine, reflux, 2 h, 82%; (b) $CNCH_2CN$, toluene, $POCl_3$, 100 °C, 83%.



Scheme 2. Reagents and conditions: (i) $POCl_3$, 130 °C, 3 h, 70%.

3. Biological evaluation

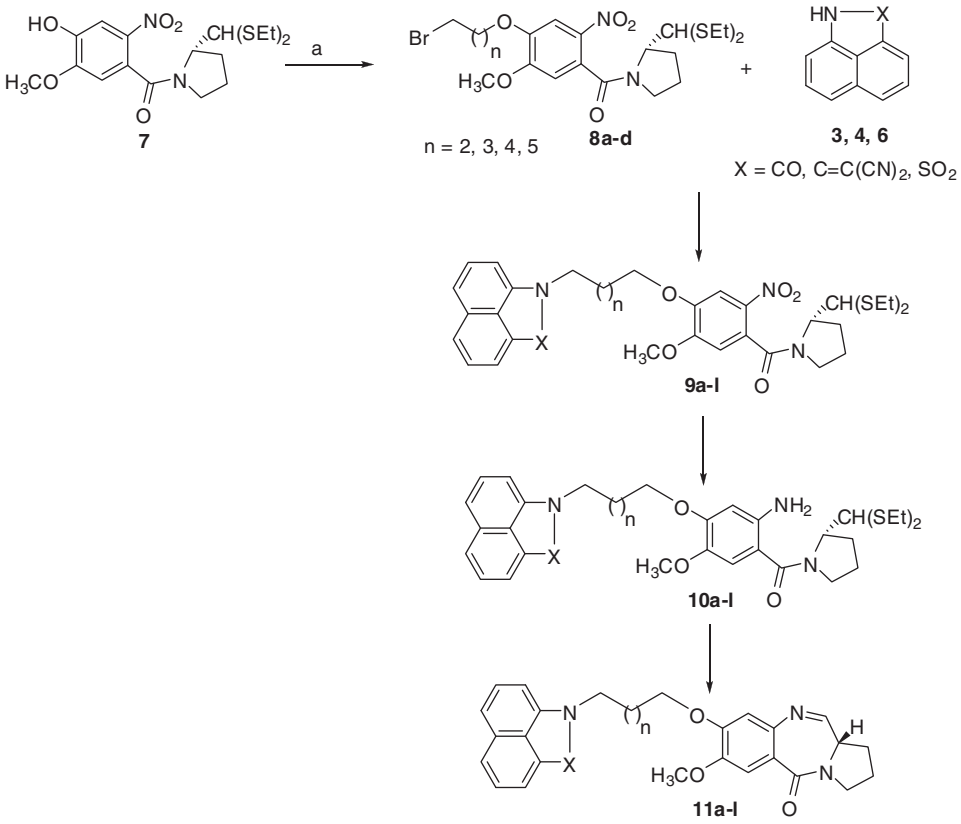
3.1. Anticancer activity

The synthesized compounds **11a–l** were evaluated for their anticancer activity in selected human cancer cell lines of lung, skin, colon and prostrate by using MTT assay method. All these new conjugates exhibit promising anticancer activity with IC_{50} values ranging from 1.05 to 36.49 μM. The positive controls, doxorubicin and DC-81 showed the IC_{50} values in the range of 0.03–2.51 μM and 0.86–1.65 μM respectively. Interestingly, among all the conjugates, compound **11l** exhibited significant activity in all the employed cell lines and is comparable to that of standards as shown in Table 1. The morphology of A549 cancer cell lines with or without treatment of compound **11l** has been shown in Figure 2.

Selective killing of cancer cells without affecting normal cell growth is an important characteristic in cancer chemotherapy. Therefore all the conjugates **11a–l** were evaluated for possible cytotoxicity towards normal cells, for example, HEK-293. The growth of HEK-293 cell line was not significantly affected by these conjugates (**11a–l**), suggesting that they selectively inhibit the growth of cancer cells.

3.2. Thermal denaturation studies

The DNA binding affinity of these new PBD conjugates has been evaluated through thermal denaturation studies with duplex-form of calf thymus DNA (CT-DNA) by using modified reported procedure.[26] The DNA-PBD solutions are incubated at 37 °C for 0 h and 18 h prior to analysis. Samples are monitored at 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller and heated at 1 °C/min in the range of 40–95 °C. DNA helix-coil transition temperatures are given by: $\Delta T_m = T_m(\text{DNA + PBD}) - T_m(\text{DNA alone})$, where the T_m value for the PBD-free CT-DNA is 69.1 ± 0.01.



Scheme 3. Reagents and conditions: (a) Acetone, anhydrous K_2CO_3, dibromo alkanes, rt, 12 h; (b) dry DMF, sodium hydride, rt, 12 h; 55–85%; (c) $SnCl_2{\cdot}2H_2O$, CH_3OH, reflux, 3 h; (d) $HgCl_2$, $CaCO_3$, CH_3CN–H_2O (4:1), 8 h, 55–75%.

Table 1
IC_{50} values[a] (in μM) for compounds **11a–l** in selected human cancer cell lines

Compound	X	*n*	A549[b]	A431[c]	Colo-205[d]	PC-3[e]
11a	CO	1	13.85 ± 0.38	3.04 ± 0.16	8.65 ± 0.49	7.67 ± 0.24
11b	CO	2	9.80 ± 0.14	1.34 ± 0.07	3.31 ± 0.15	3.23 ± 0.13
11c	CO	3	21.94 ± 2.14	3.71 ± 0.23	9.76 ± 0.24	15.45 ± 0.50
11d	CO	4	32.70 ± 2.0	3.31 ± 0.30	14.28 ± 0.35	4.89 ± 0.72
11e	C=C(CN)$_2$	1	4.86 ± 0.11	4.67 ± 0.11	6.10 ± 0.22	17.95 ± 0.21
11f	C=C(CN)$_2$	2	25.50 ± 0.34	2.50 ± 0.14	2.95 ± 0.31	14.82 ± 0.54
11g	C=C(CN)$_2$	3	5.86 ± 0.28	4.46 ± 0.16	25.93 ± 1.51	36.49 ± 4.98
11h	C=C(CN)$_2$	4	18.91 ± 0.66	1.25 ± 0.22	16.68 ± 0.93	19.31 ± 3.66
11i	SO_2	1	10.01 ± 0.83	9.56 ± 0.59	7.66 ± 0.31	9.80 ± 1.96
11j	SO_2	2	19.58 ± 0.25	3.34 ± 0.28	3.71 ± 0.24	14.90 ± 0.39
11k	SO_2	3	14.38 ± 0.39	4.67 ± 0.22	15.07 ± 0.19	9.70 ± 0.79
11l	SO_2	4	1.05 ± 0.26	1.72 ± 0.47	1.21 ± 0.20	1.52 ± 0.06
DC-81	—	—	1.11 ± 0.20	1.65 ± 0.61	0.86 ± 0.26	1.19 ± 0.34
Dox	—	—	1.02 ± 0.10	0.03 ± 0.03	1.69 ± 0.07	2.51 ± 0.10

[a] 50% Inhibitory concentration and the values are average of three individual experiments.
[b] Lung cancer.
[c] Skin cancer.
[d] Colon cancer.
[e] Prostate cancer.

Interestingly, all the compounds (**11a–l)** elevate the helix melting temperature of CT-DNA in the range of 1.1–7.6 °C. Particularly, compounds **11c**,**k** and **11l** have shown ΔT_m ranging from 1.1 to 2.1 °C at 0 h and increases upto 5.2–7.6 °C after 18 h incubation. Moreover, the naturally occurring PBD that is DC-81 exhibits a ΔT_m of 0.7 °C under similar experimental conditions as illustrated in Table 2. Vander Waals interactions are responsible for enhanced DNA binding interactions. In the present study, among all the compounds, compound **11l** has showed significant DNA binding ability due to strong hydrogen bonding interactions with DNA.

3.3. Cell cycle analysis and apoptotic changes

Many anticancer drugs interact with cells leading to cell growth arrest or cell death. To shed more light on the mechanisms responsible for the cytotoxic effect of **11l**, we examined its influence on cell cycle progression. After 48 h of treatment with the compound **11l,** it was observed that the percentage of cells in G0/G1 phase was decreased and accumulation of cells in subG1 phase increased in dose-dependent manner, which indicates the onset of apoptosis[27] (Fig. 3).

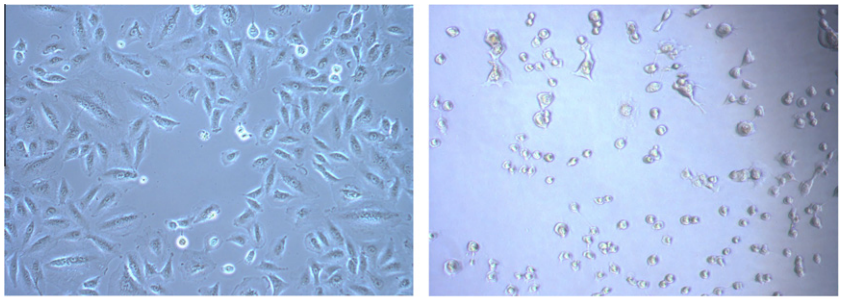


Figure 2. Effects of compound **11l** on cell morphological changes.

Table 2
Thermal denaturation data for benzo[*c*,*d*]indol-2(1*H*)-one-PBD conjugates (**11a–l**) with calf thymus (CT) DNA

Compound	[PBD]:[DNA] molar ratio[b]	(ΔT_m, °C)[a] after incubation at 37 °C for	
		0 h	18 h
11a	1:5	3.1	3.2
11b	1:5	1.1	4.0
11c	1:5	2.1	5.2
11d	1:5	1.1	2.1
11e	1:5	2.8	3.4
11f	1:5	1.3	2.7
11g	1:5	1.3	2.8
11h	1:5	1.3	2.0
11i	1:5	2.0	4.9
11j	1:5	1.1	3.9
11k	1:5	1.1	5.6
11l	1:5	1.5	7.6
DC-81	1:5	0.3	0.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.1 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ±0.1–0.2 °C.
[b] For a 1:5 molar ratio of [PBD]/[DNA], where CT-DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01].

In addition, we have analyzed the cellular effects of the compound (**11l**) on nuclear condensation by Hoechst staining. Cells treated with compound **11l** showed significant effect on nuclear condensation compared with standard DC-81 (Fig. 4). These results clearly demonstrated that the compound **11l** is effective in inducing cellular apoptosis.

3.4. Annexin V-FITC for apoptosis

The apoptotic effect of the compound **11l** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis. After 48 h treatment with compound **11l** (1 μM and 2 μM), it was observed that this compound showed significant apoptosis against A549 cells as shown in Figure 5. Results indicated that compound **11l** showed 57.49% and 68.47% apoptosis at 1 and 2 μM respectively, whereas the standard DC-81 showed 56.83% apoptosis at 1 μM concentration.

3.5. Caspase-3 activity

Caspases, or cysteine-aspartic proteases, are a family of cysteine proteases, which are crucial mediators of apoptosis. Among them, caspase-3 is the best understood in the mammalian caspases in terms of its specificity and role in apoptosis. Caspase-3 is also required for some typical hallmarks of apoptosis.[28] A549 cells were treated with **11l** (1 μM and 2 μM) along with the positive control DC-81 and were examined for the activation of caspase-3 activity. Results indicated that there was nearly 4 to 8-fold induction in caspase-3 levels compared to control (Fig. 6).

3.6. DNA fragmentation

DNA laddering was carried out in order to elucidate the mode of action of the compound especially for their ability to induce oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis.[29,30] During apoptosis DNA is cleaved into small fragments by endonucleases. These fragments can be observed by gel electrophoresis as ladders. A549 cells were treated with compound **11l** at 1 and 2 μM concentrations for 48 h and DNA was isolated from these cells. The DNA was run on 2% agarose gel electrophoresis after staining with ethydium bromide under UV illumination. It is observed that compound **11l** produced significant DNA fragmentation (Fig. 7), which is indicative of apoptosis.

4. Conclusion

In the present study, a series of benzo[*c*,*d*]indol-2(1*H*)one-PBD conjugates have been synthesized. All these new PBD conjugates have showed better DNA binding ability when compared to standard DC-81. Particularly, compound **11l** showed ΔT_m values ranging from 1.5 to 7.6 °C. Whereas, some of the PBD conjugates previously synthesized showed ΔT_m values in the range of 1.0–2.3 °C (quinazolone linked PBDs),[31] 1.1–5.8 °C (2,5-diaryloxadiazole-PBD conjugates),[32] 1.2–7.1 °C (aniline substituted pyrimidine linked PBD conjugates).[33] These compounds showed the anti cancer activity against the four cancer cell lines. Moreover, from

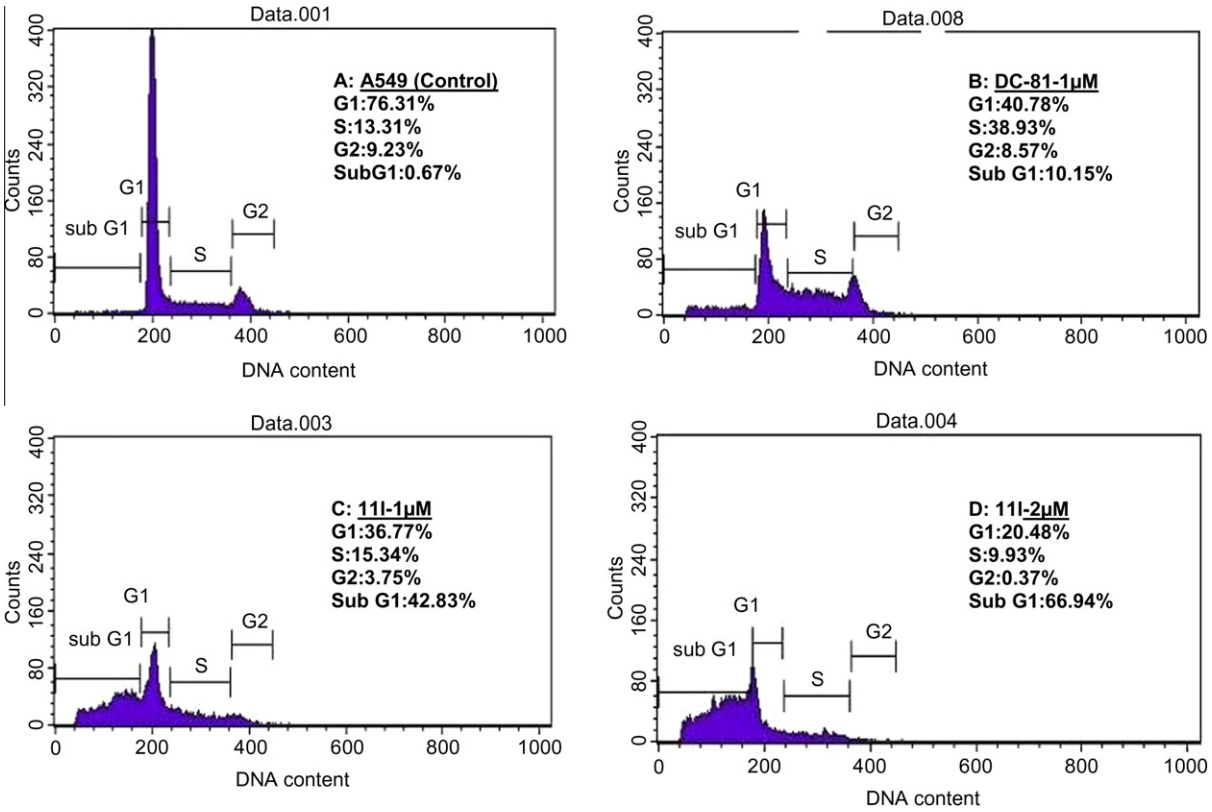


Figure 3. Cell cycle analysis of **11l** on A549 cells. (A) A549 (control cells), (B) DC-81 (1 μM), (C) **11l** (1 μM), and (D) **11l** (2 μM).

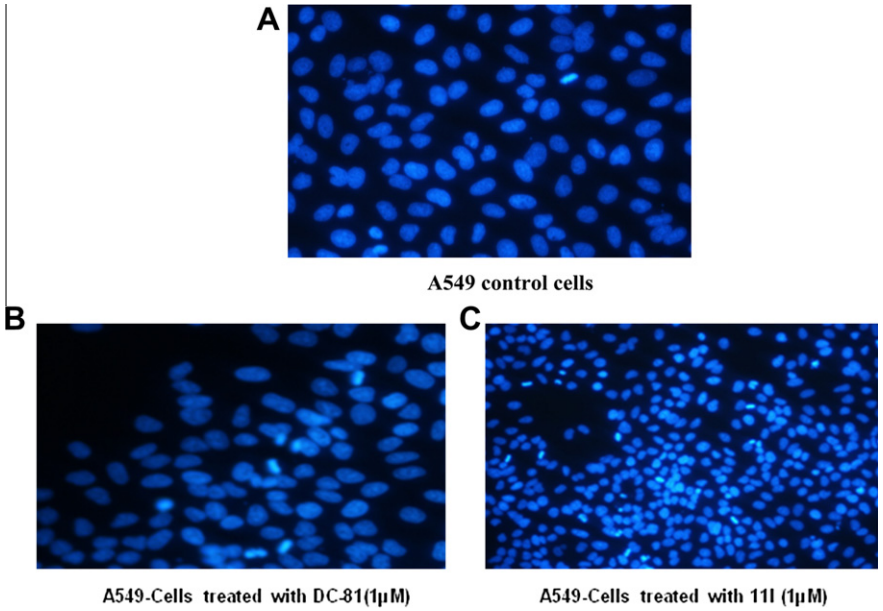


Figure 4. Hoechst staining. (A) Control, (B) cells treated with DC-81 (2 μM), and (C) cells treated with **11l** (2 μM).

the MTT assay it was observed that the compound **11l** is more effective anti cancer agent than the DC-81 in A549, lung cancer cell line. The FACS analysis suggested that the cell death is due to the arrest in SubG1 phase indicating that the compound **11l** has cell cycle regulatory properties and probably leading to caspase-3 dependent apoptotic cell death and it was confirmed by caspase-3 activity assay. This was further confirmed by DNA fragmentation, Hoechst staining as well as Annexin V-FITC assay. Moreover, these compounds **11a–l** did not affect the normal cell line (HEK-293) which is the most important characteristic property for cancer chemotherapy. Based on these observations, compound (**11l**) could be considered as important lead compound for potential application in anticancer chemotherapy.

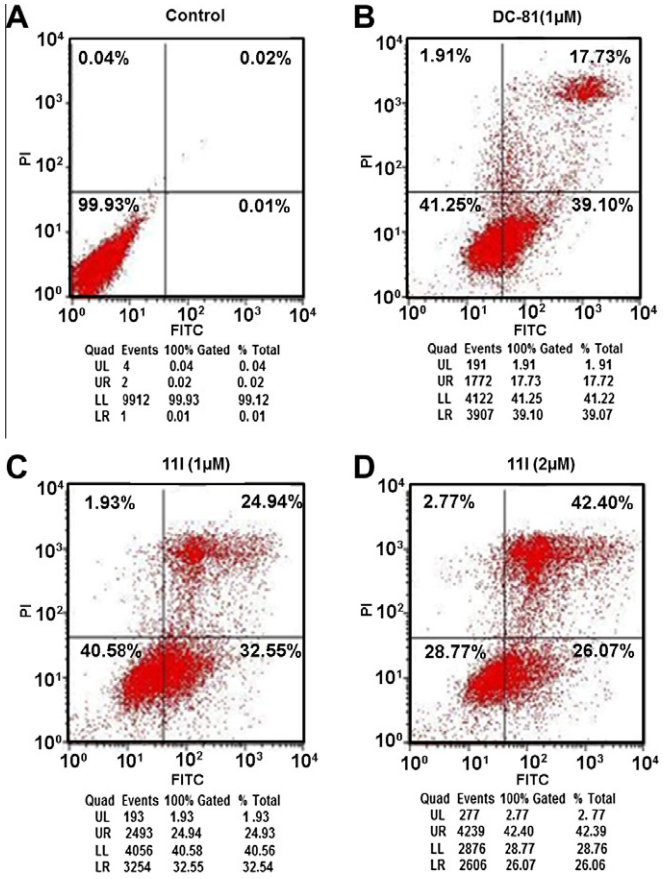


Figure 5. Annexin V-FITC. (A) A549 (control cells), (B) DC-81 (1 μM), (C) **11l** (1 μM), and (D) **11l** (2 μM).

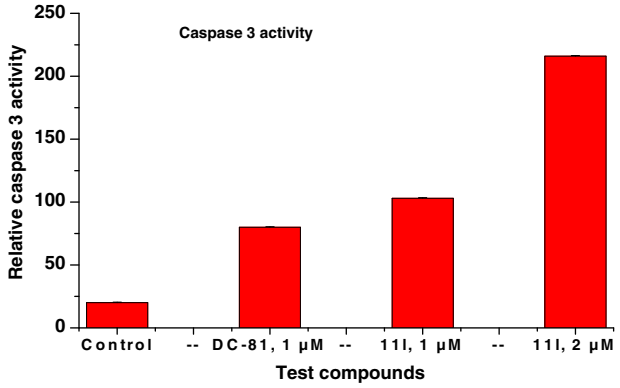


Figure 6. Caspase 3 activity assay.

5. Experimental

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthew Company, Ward Hill, MA, USA) and were used without purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silicagel. ^{1}H NMR spectra were recorded on Gemini Varian VXR-unity (400 and 500 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micro mass, Quattro LC using ESI$^+$ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High-resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Melting points were determined with an Electrothermal melting point apparatus, and are uncorrected. Optical rotations are measured on Horiba, high sensitive polarimeter, SEPA-300.

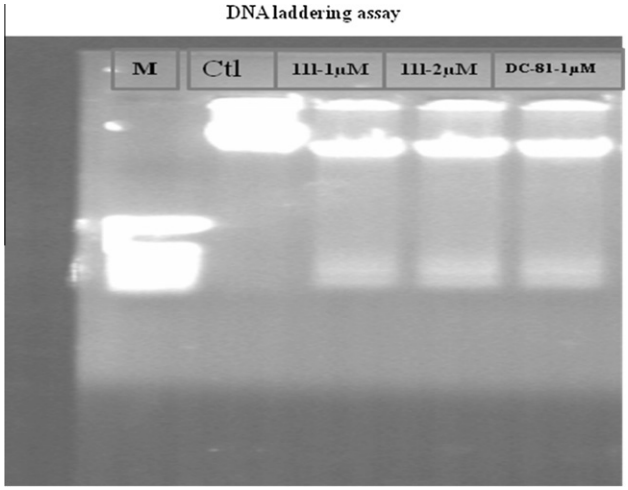


Figure 7. DNA laddering assay: lane-1: marker, lane-2: control, lane-3: **11l**-1 μM, lane-4: **11l**-2 μM, lane-5: DC-81–1 μM.

5.1. Benzo[*c,d*]indol-2(1*H*)-one (3)

Napthalic anhydride (10 mmol, 1.98 g), hydroxylamine hydrochloride (10 mmol, 0.690 mg) and dry pyridine (5 mL) were added under reflux for 1 h. Heating was discontinued and toluene-*p*-sulphonyl chloride (5 g) was added portion wise to cause controlled boiling. Finally, heating was resumed for 1 h, and the hot mixture was poured into water (30 ml). The crystalline precipitate was collected, washed with aqueous alkali and water. The crystals were boiled with water (15 mL) and ethanol (5 mL) containing sodium hydroxide (5 g) for 2 h, during the second of which, ethanol was allowed to distil out. The solution was acidified with concentrated hydrochloric acid (0.3 mL), carbon dioxide being evolved and yellow crystals deposited. Next day, the crystals were washed, and dried at 100 °C, to give light yellow needles (1.38 g, 82%). Mp 172–178 °C; ^{1}H NMR (DMSO, 300 MHz): δ 8.05 (d, 1H, J = 6.7 Hz), 8.01 (d, 1H, J = 8.3 Hz), 7.75–7.70 (m, 1H), 7.53 (d, 1H, J = 8.3 Hz), 7.40 (dd, 1H, J = 7.5, 6.7 Hz), 6.94 (d, 1H, J = 6.7 Hz); MS (EI): m/z 169 (M^+).

5.2. 2-(1,2-Dihydrobenzo[*c,d*]indol-2-yliden)malononitrile (4)

Benzo[*c,d*]indol-2(1*H*)-one (1.69 g, 10 mmol) and malononitrile (0.6 g, 10 mmol) were dissolved in 15 mL toluene. $POCl_3$ (1.1 mL) was added dropwise while the mixture was stirring. Then temperature of the reaction mixture was raised to 100 °C. After 4 h, the mixture was cooled and added with 10 mL methanol, filtered, and dried, separated on silica gel chromatography by using dichloromethane as solvent to afford the product **4** (1.82 g, 83%). Mp >300 °C; ^{1}H NMR (DMSO, 300 MHz): δ 8.55 (d, 1H, J = 8.0 Hz), 8.17 (d, 1H, J = 8.0 Hz), 7.75–7.70 (m, 1H), 7.68 (d, 1H, J = 8.0 Hz), 7.42 (dd, 1H, J = 7.5, 6.7 Hz), 6.96 (d, 1H, J = 6.8 Hz), 5.30 (s, 1H); MS (EI): m/z 217 (M^++H).

5.3. 1,2-Dihydro-1λ⁶-naphtho[1,8-*cd*]isothiazole-1,1-dione 6

Five grams of the potassium salt of 1,8 naphthyl amine sulphonic acid were pulverized and placed in a flask containing three times that weight of $POCl_3$. After attaching a reflux condenser the whole was heated in an oil bath to 130 °C. At 100 °C the offensive vapors of hydrochloric acid are given off and must be led into flue. To hasten the reaction the flask is shaken repeatedly. After the irritating vapors have ceased to be given off the heating is continued until the contents of the flask ceases to form a dyestuff when diazotized and developed. As a rule the reaction is complete after 3 h. The removal of the excess $POCl_3$ requires some care because of the mud like consistency of the contents of the flask. Finally the contents of the flask are poured into ice bath. The grey product is filtered off by means of a suction pump and greater part of the phosphoric acid removed by repeated washing. The crude product was recrystallised from benzene after through drying. Mp 175–180 °C; ^{1}H NMR (DMSO, 300 MHz): δ 8.62 (d, 1H, J = 8.0 Hz), 8.12 (d, 1H, J = 8.0 Hz), 7.75–7.74 (m, 1H), 7.61 (d, 1H, J = 8.0 Hz), 7.32 (dd, 1H, J = 7.5, 6.7 Hz), 6.90 (d, 1H, J = 6.8 Hz), 5.36 (s, 1H); MS (EI): m/z 206 (M^++H).

5.4. (2*S*)-*N*-[4-(3-Bromopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (8a)

To a solution of compound **7** (400 mg, 1 mmol) in dry acetone (15 ml) was added, anhydrous K_2CO_3 (553 mg, 4 mmol), 1,3-dibromopropane (256 mg, 1.2 mmol) and the mixture was stirred at reflux temperature for 48 h. The reaction was monitored by TLC using EtOAc–hexane (1:1). After completion of the reaction as indicated by TLC, K_2CO_3 was removed by filtration and the solvent was evaporated under reduced pressure, diluted with water and extracted with ethyl acetate. The combined organic phases were dried over Na_2SO_4 and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using ethyl acetate and hexane (2:3) to afford compound **8a** as yellow liquid (500 mg, 96%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.65 (s, 1H), 6.80 (s, 1H), 4.86 (d, 1H, J = 4.3 Hz), 4.72–4.61 (m, 1H), 4.25 (t, 2H, J = 6.0 Hz), 3.95 (s, 3H), 3.65–3.55 (m, 2H), 3.31–3.15 (m, 2H), 2.90–2.60 (m, 4H), 2.40–1.70 (m, 6H), 1.45–1.21 (m, 6H); MS (ESI): m/z 521 $(M+1)^+$.

5.5. (2*S*)-*N*-[4-(4-Bromobutyloxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (8b)

The compound **8b** was prepared according to the method described for compound **8a** by employing **7** (400 mg, 1 mmol) and 1,4-dibromobutane (268 mg, 1.2 mmol) to afford the compound **8b** (492 mg, 92%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.65 (s, 1H), 6.80 (s, 1H), 4.87 (d, 1H, J = 4.3 Hz), 4.71–4.60 (m, 1H), 4.15 (t, 2H, J = 6.0 Hz), 3.95 (s, 3H), 3.59–3.42 (m, 2H), 3.30–3.15 (m, 2H), 2.85–2.65 (m, 4H), 2.40–1.60 (m, 8H), 1.40–1.21 (m, 6H); MS (ESI): m/z 536 $(M+1)^+$.

5.6. (2*S*)-*N*-[4-(5-Bromopentyloxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethylthioacetal (8c)

The compound **8c** was prepared according to the method described for compound **8a** by employing **7** (400 mg, 1 mmol) and 1,5-dibromopentane (270 mg, 1.2 mmol) to afford the compound **8c** (522 mg, 94%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.64 (s, 1H), 6.80 (s, 1H), 4.83 (d, 1H, J = 4.3 Hz), 4.72–4.62 (m, 1H), 4.15–4.05 (t, 2H, J = 6.0 Hz), 3.95 (s, 3H), 3.51–3.43 (m, 2H), 3.30–3.16 (m, 2H), 2.85–2.65 (m, 4H), 2.40–1.61 (m, 10H), 1.41–1.23 (m, 6H); MS (ESI): m/z 550 $(M+1)^+$.

5.7. 4-[(6-Bromohexyl)oxy]-5-methoxy-2-nitrophenyl2-[di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylmethanone (8d)

The compound **8d** was prepared according to the method described for compound **8a** by employing **7** (400 mg, 1 mmol) and 1,6-dibromopentane (292 mg, 1.2 mmol) to afford the compound **8d** (507 mg, 90%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.63 (s, 1H), 6.80 (s, 1H), 4.83 (d, 1H, J = 4.3 Hz), 4.74–4.60 (m, 1H), 4.14–4.03 (t, 2H, J = 6.0 Hz), 3.95 (s, 3H), 3.51–3.43 (m, 2H), 3.30–3.16 (m, 2H), 2.85–2.65 (m, 4H), 2.40–1.61 (m, 12H), 1.41–1.23 (m, 6H); MS (ESI): m/z 564 $(M+1)^+$.

5.8. 1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]propyl-1,2-dihydrobenzo[*c*,*d*]indol-2-one (9a)

To a solution of 2*S*-*N*-[4-(3-bromopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**8a**) (521 mg, 1.0 mmol) in dry DMF (10 mL) was added sodium hydride (5.0 mmol) and compound **3** (169 mg, 1.0 mmol). The reaction mixture was stirred at room temperature for 24 h and the reaction was monitored by TLC using ethyl acetate–hexane (60%) as a solvent system. After the completion of reaction ice was added to the reaction mixture followed by extraction with ethyl acetate (3 × 20 mL) and finally washed with brine solution. Then the solvent was evaporated under vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate–hexane (70%) as a solvent system to obtain the pure product **9a** as a yellow solid. Yield 427 mg, 70%; mp 70–75 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.05 (d, 1H, J = 6.7 Hz), 8.01 (d, 1H, J = 8.3 Hz), 7.75–7.69 (m, 1H), 7.60 (s,1H), 7.53 (d, 1H, J = 8.3 Hz), 7.40 (dd, 1H, J = 7.5 Hz), 6.94 (d, 1H, J = 6.7 Hz), 6.80 (d, 1H, J = 7.5 Hz), 4.87 (d, 1H, J = 3.7 Hz), 4.73–4.66 (m, 1H), 4.20–4.12 (m, 3H), 3.88 (s, 3H), 3.30–3.15 (m, 2H), 2.86–2.65 (m, 6H), 2.42–2.20 (m, 3H), 1.87–1.72 (m, 1H), 1.38–1.31 (q, 6H, J = 7.5 Hz), 1.29–1.19 (m, 1H); ESIMS: m/z 610 (M^+).

5.9. 1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]butyl-1,2-dihydrobenzo[*c*,*d*]indol-2-one (9b)

The compound **9b** was prepared according to the method described for compound **9a** by employing **8b** (536 mg, 1 mmol) and compound **3** (169 mg, 1.0 mmol) to afford the compound **9b** (386 mg, 62%). Mp 71–76 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 1H, J = 6.7 Hz), 8.01 (d, 1H, J = 7.5 Hz), 7.72 (t, 1H, J = 8.3 Hz), 7.64 (s,1H), 7.54 (d, 1H, J = 8.3 Hz), 7.47 (t, 1H, J = 6.7 Hz), 6.94 (d, 1H, J = 6.7 Hz), 6.80 (s, 1H), 4.87 (d, 1H, J = 3.7 Hz), 4.73–4.66 (m, 1H), 4.16–4.08 (m, 2H), 4.08–4.02 (s, 2H), 3.91 (s, 3H), 3.32–3.17 (m, 2H), 2.86–2.65 (m, 4H), 2.36–2.22 (m, 1H), 2.13–1.90 (m, 6H), 1.33 (q, 6H, J = 7.5 Hz), 1.28–1.18 (m, 1H); ESIMS: m/z 624 (M^+).

5.10. 1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]pentyl-1,2-dihydrobenzo[*c*,*d*]indol-2-one (9c)

The compound **9c** was prepared according to the method described for compound **9a** by employing **8c** (549 mg, 1 mmol) and compound **3** (169 mg, 1.0 mmol) to afford the compound **9c** (433 mg, 68%). Mp 69–74 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.05 (d, 1H, J = 6.9 Hz), 8.02 (d, 1H, J = 8.1 Hz), 7.72 (dd, 1H, J = 6.9,

6.7 Hz), 7.62 (s,1H), 7.52 (d, 1H, *J* = 8.4 Hz), 7.46 (dd, 1H, *J* = 6.9, 6.7 Hz), 6.92 (d, 1H, *J* = 6.7 Hz), 6.79 (s, 1H), 4.86 (d, 1H, *J* = 3.7 Hz), 4.73–4.67 (m, 1H), 4.12–4.02 (m, 2H), 3.98 (t, 2H, *J* = 6.9 Hz), 3.89 (s, 3H), 3.33–3.16 (m, 2H), 2.87–2.64 (m, 4H), 2.01–1.82 (m, 6H), 1.69–1.54 (m, 5H), 1.34 (q, 6H, *J* = 7.3 Hz); ESIMS: *m/z* 638 (M^++1).

5.11. 1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]hexyl-1,2-dihydrobenzo[*c*,*d*]indol-2-one (9d)

The compound **9d** was prepared according to the method described for compound **9a** by employing **8d** (563 mg, 1 mmol) and compound **3** (169 mg, 1.0 mmol) to afford the compound **9d** (371 mg, 57%). Mp 68–73 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.07 (d, 1H, *J* = 6.9 Hz), 8.03 (d, 1H, *J* = 8.1 Hz), 7.71 (t, 1H, *J* = 7.1 Hz), 7.64 (s, 1H), 7.55 (d, 1H, *J* = 8.3 Hz), 7.46 (t, 1H, *J* = 6.9 Hz), 6.93 (d, 1H, *J* = 6.7 Hz), 6.80 (s, 1H), 4.88 (d, 1H, *J* = 3.7 Hz), 4.74–4.68 (m, 1H), 4.06 (t, 2H, *J* = 6.4 Hz), 3.97–3.92 (m, 3H), 3.91 (s, 3H), 3.32–3.18 (m, 2H), 2.86–2.65 (m, 4H), 2.37–2.01 (m, 2H), 1.94–1.77 (m, 4H), 1.60–1.50 (m, 5H), 1.33 (q, 6H, *J* = 7.3 Hz); ESIMS: *m/z* 652 (M^+).

5.12. 2-(1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]propyl-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden)malononitrile (9e)

The compound **9e** was prepared according to the method described for compound **9a** by employing **8a** (520 mg, 1 mmol) and compound **4** (216 mg, 1.0 mmol) to afford the compound **9e** (407 mg, 62%). Mp 60–65 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.08 (d, 1H, *J* = 6.7 Hz), 8.02 (d, 1H, *J* = 8.3 Hz), 7.78–7.71 (m, 1H), 7.60 (s, 1H), 7.53 (d, 1H, *J* = 8.3 Hz), 7.44 (dd, 1H, *J* = 7.5 Hz), 6.96 (d, 1H, *J* = 6.7 Hz), 6.82 (d, 1H, *J* = 7.5 Hz), 4.87 (d, 1H, *J* = 3.7 Hz), 4.73–4.66 (m, 1H), 4.20–4.15 (m, 3H), 3.89 (s, 3H), 3.30–3.15 (m, 2H), 2.86–2.62 (m, 6H), 2.42–2.25 (m, 3H), 1.87–1.74 (m, 1H), 1.38–1.31 (q, 6H, *J* = 7.5 Hz), 1.29–1.19 (m, 1H); ESIMS: *m/z* 658 (M^++1).

5.13. 2-(1-3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]butyl-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden)malononitrile (9f)

The compound **9f** was prepared according to the method described for compound **9a** by employing **8b** (536 mg, 1 mmol) and compound **4** (216 mg, 1.0 mmol) to afford the compound **9f** (437 mg, 65%). Mp 62–67 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 1H, *J* = 6.7 Hz), 8.02 (d, 1H, *J* = 7.5 Hz), 7.74 (t, 1H, *J* = 8.3 Hz), 7.64 (s, 1H), 7.57 (d, 1H, *J* = 8.3 Hz), 7.48 (t, 1H, *J* = 6.7 Hz), 6.94 (d, 1H, *J* = 6.7 Hz), 6.80 (s, 1H), 4.88 (d, 1H, *J* = 3.7 Hz), 4.73–4.68 (m, 1H), 4.16–4.09 (m, 2H), 4.08–4.05 (s, 2H), 3.91 (s, 3H), 3.32–3.19 (m, 2H), 2.86–2.65 (m, 4H), 2.36–2.26 (m, 1H), 2.13–1.94 (m, 6H), 1.35 (q, 6H, *J* = 7.5 Hz), 1.28–1.19 (m, 1H); ESIMS: *m/z* 672 (M^+).

5.14. 2-(1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]pentyl-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden)malononitrile (9g)

The compound **9g** was prepared according to the method described for compound **9a** by employing **8c** (549 mg, 1 mmol) and compound **4** (216 mg, 1.0 mmol) to afford the compound **9g** (466 mg, 68%). Mp 59–64 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.07 (d, 1H, *J* = 6.9 Hz), 8.03 (d, 1H, *J* = 8.1 Hz), 7.75 (dd, 1H, *J* = 6.9, 6.7 Hz), 7.62 (s, 1H), 7.54 (d, 1H, *J* = 8.4 Hz), 7.46 (dd, 1H, *J* = 6.9, 6.7 Hz), 6.94 (d, 1H, *J* = 6.7 Hz), 6.79 (s, 1H), 4.87 (d, 1H, *J* = 3.7 Hz), 4.73–4.67 (m, 1H), 4.12–4.02 (m, 2H), 3.99 (t, 2H, *J* = 6.9 Hz), 3.89 (s, 3H), 3.33–3.18 (m, 2H), 2.87–2.66 (m, 4H), 2.01–1.80 (m, 6H), 1.69–1.54 (m, 5H), 1.36 (q, 6H, *J* = 7.5 Hz); ESIMS: *m/z* 687 (M^++1).

5.15. 2-(1–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]hexyl-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden)malononitrile (9h)

The compound **9h** was prepared according to the method described for compound **9a** by employing **8d** (563 mg, 1 mmol) and compound **4** (216 mg, 1.0 mmol) to afford the compound **9h** (413 mg, 59%). Mp 62–66 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 1H, *J* = 6.9 Hz), 8.03 (d, 1H, *J* = 8.1 Hz), 7.72 (t, 1H, *J* = 7.1 Hz), 7.64 (s, 1H), 7.56 (d, 1H, *J* = 8.3 Hz), 7.44 (t, 1H, *J* = 6.9 Hz), 6.93 (d, 1H, *J* = 6.7 Hz), 6.81 (s, 1H), 4.87 (d, 1H, *J* = 3.7 Hz), 4.73–4.68 (m, 1H), 4.06 (t, 2H, *J* = 6.4 Hz), 3.98–3.94 (m, 3H), 3.91 (s, 3H), 3.32–3.18 (m, 2H), 2.86–2.63 (m, 4H), 2.37–2.01 (m, 2H), 1.94–1.76 (m, 4H), 1.60–1.50 (m, 5H), 1.32 (q, 6H, *J* = 7.3 Hz); ESIMS: *m/z* 700 (M^+).

5.16. 2–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]propyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (9i)

The compound **9i** was prepared according to the method described for compound **9a** by employing **8a** (520 mg, 1 mmol) and compound **6** (205 mg, 1.0 mmol) to afford the compound **9i** (413 mg, 64%). Mp 54–59 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.08 (d, 1H, *J* = 8.3 Hz), 7.96 (d, 1H, *J* = 7.3 Hz), 7.67 (s, 1H), 7.52–7.42 (m, 2H), 6.85 (s, 1H), 6.81 (dd, 1H, *J* = 6.4 Hz), 4.87 (d, 1H, *J* = 3.7 Hz), 4.75–4.67 (m, 1H), 4.28 (t, 2H, *J* = 5.8 Hz), 4.17–4.09 (m, 2H), 3.98 (s, 3H), 3.34–3.17 (m, 2H), 2.88–2.67 (m, 4H), 2.52–2.42 (m, 2H), 2.35–2.21 (m, 1H), 2.12–1.72 (m, 3H), 1.35 (q, 6H, *J* = 7.3 Hz); ESIMS: *m/z* 646 (M^+).

5.17. 2–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]butyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (9j)

The compound **9j** was prepared according to the method described for compound **9a** by employing **8b** (536 mg, 1 mmol) and compound **6** (205 mg, 1.0 mmol) to afford the compound **9j** (396 mg, 60%). Mp 56–61 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.09 (d, 1H, *J* = 8.1 Hz), 7.96 (d, 1H, *J* = 7.1 Hz), 7.76 (t, 1H, *J* = 7.3 Hz), 7.67 (s,1H), 7.54 (t, 1H, *J* = 7.3 Hz), 7.48 (d, 1H, *J* = 8.3 Hz), 6.82 (s, 1H), 6.79 (d, 1H, *J* = 6.9 Hz), 4.88 (d, 1H, *J* = 3.7 Hz), 4.75–4.67 (m, 1H), 4.18 (t, 2H, *J* = 5.8 Hz), 3.99–3.97 (m, 2H), 3.94 (s, 3H), 3.34–3.18 (s, 2H), 2.88–2.66 (s, 4H), 2.13–1.92 (m, 5H), 1.60 (s, 3H), 1.36 (q, 6H, *J* = 7.3 Hz); ESIMS: *m/z* 660 (M^+).

5.18. 2–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]pentyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (9k)

The compound **9k** was prepared according to the method described for compound **9a** by employing **8c** (549 mg, 1 mmol) and compound **6** (205 mg, 1.0 mmol) to afford the compound **9k** (458 mg, 68%). Mp 54–59 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 1H, *J* = 7.5 Hz), 7.95 (d, 1H, *J* = 6.7 Hz), 7.75 (t, 1H, *J* = 7.5 Hz), 7.66 (s, 1H), 7.54 (t, 1H, *J* = 7.5 Hz), 7.44 (d, 1H, *J* = 8.3 Hz), 6.81 (s, 1H), 6.73 (d, 1H, *J* = 6.7 Hz), 4.87 (d, 1H, *J* = 4.5 Hz), 4.74–4.67 (m, 1H), 4.17–4.08 (m, 2H), 3.92 (s, 3H), 3.87 (t, 2H, *J* = 6.7 Hz), 3.32–3.18 (m, 2H), 2.86–2.65 (m, 4H), 2.13–1.92 (m, 7H), 1.84–1.68 (m, 3H), 1.34 (q, 6H, *J* = 7.5 Hz). ESIMS: *m/z* 675 (M^++1).

5.19. 2–3-[4-((2*S*)-2-[Di(ethylsulfanyl)methyl]tetrahydro-1*H*-1-pyrrolylcarbonyl)-2-methoxy-5-nitrophenoxy]hexyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (9l)

The compound **9l** was prepared according to the method described for compound **9a** by employing **8d** (563 mg, 1 mmol) and compound **6** (205 mg, 1.0 mmol) to afford the compound **9l** (440 mg, 64%). Mp 62–66 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 1H, J = 8.3 Hz), 7.93 (d, 1H, J = 6.7 Hz), 7.74 (t, 1H, J = 7.5 Hz), 7.62 (s, 1H), 7.5 (t, 1H, J = 7.5 Hz), 7.40 (d, 1H, J = 9.0 Hz), 6.77 (s, 1H), 6.69 (d, 1H, J = 6.7 Hz), 4.83 (d, 1H, J = 3.7 Hz), 4.71–4.63 (m, 1H), 4.09 (t, 2H, J = 6.0 Hz), 3.93 (s, 3H), 3.83 (t, 2H, J = 6.7 Hz), 3.31–3.16 (m, 2H), 2.87–2.63 (m, 4H), 2.33–2.22 (m, 1H), 2.12–1.74 (m, 6H), 1.66–1.56 (m, 3H), 1.51–1.48 (m, 2H), 1.34 (q, 6H, J = 7.5 Hz); ESIMS: m/z 688 (M^+).

5.20. (11a*S*)-7-Methoxy-8-[3-(2-oxo-1,2-dihydrobenzo[*c*,*d*]indol-1-yl)propoxy]-2,3,5,11*a*-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-5-one (11a)

A solution of amino thioacetal **10a** (579 mg, 1 mmol), $HgCl_2$ (1.19 mg, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in acetonitrile–water (4:1) was stirred slowly at rt for 24 h 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 satd aq $NaHCO_3$ (2 × 20 mL), brine (2 × 20 mL) and the combined organic phase was dried (Na_2SO_4). The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (2% MeOH–$CHCl_3$) to afford the compound **11a** as a white solid; (255 mg, 56% yield). Mp 85–90 °C; $[\alpha]_D^{27}$ +49.8 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (d, 1H, J = 6.7 Hz), 8.01 (d, 1H, J = 8.3 Hz), 7.70 (t, 1H, J = 7.5 Hz), 7.61 (d, 1H, J = 4.5 Hz), 7.47 (d, 2H, J = 8.3 Hz), 7.37 (t, 1H, J = 6.7 Hz), 6.98 (d, 1H, J = 6.7 Hz), 6.72 (s, 1H), 4.20–4.03 (m, 2H), 3.93 (s, 3H), 3.88–3.77 (m, 1H), 3.71–3.66 (m, 1H), 3.62–3.53 (m, 1H), 2.41–2.23 (m, 4H), 2.09–1.98 (m, 2H), 1.83–1.66 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 168.17, 162.33, 150.28, 147.67, 140.48, 139.34, 138.91, 130.76, 129.99, 128.52, 126.46, 124.19, 120.23, 111.42, 105.07, 65.79, 56.02, 53.61, 46.60, 36.99, 29.49, 28.27, 24.08; ESIMS: m/z 456 (M^++1).

5.21. (11a*S*)-7-Methoxy-8-[3-(2-oxo-1,2-dihydrobenzo[*c*,*d*]indol-1-yl)butoxy]-2,3,5,11*a*-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-5-one (11b)

The compound **11b** was prepared according to the method described for the compound **11a**, employing the compound **10b** (593 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11b** as a white solid (282 mg, 60% yield). Mp 86–91 °C; $[\alpha]_D^{27}$ +47.6 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.02 (d, 1H, J = 6.9 Hz), 7.98 (d, 1H, J = 8.1 Hz), 7.69 (t, 1H, J = 5.8 Hz), 7.59 (d, 1H, J = 4.1 Hz), 7.49–7.36 (m, 3H), 6.92 (d, 1H, J = 6.6 Hz), 6.73 (s, 1H), 4.18–4.06 (m, 2H), 4.02 (t, 2H, J = 6.6 Hz), 3.91 (s, 3H), 3.85–3.74 (m, 1H), 3.71–3.66 (m, 1H), 3.61–3.49 (m, 1H), 2.35–2.25 (m, 2H), 2.09–1.90 (m, 4H), 1.64–1.45 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 164.57, 162.37, 140.48, 130.73, 129.02, 128.70, 128.58, 128.43, 126.52, 125.03, 124.42, 124.19, 120.20, 120.11, 111.36, 110.40, 105.28, 105.08, 68.27, 55.96, 53.61, 46.57, 39.74, 29.49, 26.18, 25.30, 24.86; ESIMS: m/z 470 (M^+).

5.22. (11a*S*)-7-Methoxy-8-[3-(2-oxo-1,2-dihydrobenzo[*c*,*d*]indol-1-yl)pentoxy]-2,3,5,11*a*-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-5-one (11c)

The compound **11c** was prepared according to the method described for the compound **11a**, employing the compound **10c** (607 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11c** as a white solid (280 mg, 58% yield). Mp 73–78 °C; $[\alpha]_D^{27}$ +46.2 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.02 (d, 1H, J = 6.7 Hz), 7.95 (d, 1H, J = 7.5 Hz), 7.68 (t, 1H, J = 6.7 Hz), 7.58 (d, 1H, J = 3.7 Hz), 7.48–7.40 (m, 3H), 6.87 (d, 1H, J = 6.7 Hz), 6.70 (s, 1H), 4.10–3.95 (m, 2H), 3.93 (t, 2H, J = 6.7 Hz), 3.87 (s, 3H), 3.82–3.74 (m, 1H), 3.70–3.65 (m, 1H), 3.59–3.50 (m, 1H), 2.33–2.26 (m, 2H), 2.08–1.98 (m, 2H), 1.96–1.83 (m, 4H), 1.66–1.56 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 164.57, 162.28, 150.71, 147.71, 140.55, 139.93, 130.68, 129.04, 128.57, 128.42, 126.62, 125.06, 124.15, 120.13, 120.05, 111.51, 110.38, 104.91, 68.62, 56.05, 53.63, 46.58, 40.04, 29.55, 28.56, 24.12, 23.39; ESIMS: m/z 484 (M^+).

5.23. (11a*S*)-7-Methoxy-8-[3-(2-oxo-1,2-dihydrobenzo[*c*,*d*]indol-1-yl)hexoxy]-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (11d)

The compound **11d** was prepared according to the method described for the compound **11a**, employing the compound **10d** (621 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11d** as a white solid (314 mg, 65% yield). Mp 85–90 °C; $[\alpha]_D^{27}$ +45.9 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (d, 1H, J = 6.7 Hz), 7.96 (d, 1H, J = 7.5 Hz), 7.67 (t, 1H, J = 6.7 Hz), 7.59 (d, 1H, J = 3.7 Hz), 7.48–7.40 (m, 3H), 6.88 (d, 1H, J = 6.7 Hz), 6.70 (s, 1H), 4.10–3.97 (m, 2H), 3.92 (t, 2H, J = 6.7 Hz), 3.87 (s, 3H), 3.82–3.74 (m, 1H), 3.70–3.65 (m, 1H), 3.59–3.50 (m, 1H), 2.33–2.26 (m, 2H), 2.09–1.97 (m, 4H), 1.96–1.83 (m, 4H), 1.67–1.54 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 164.59, 162.29, 150.51, 147.74, 140.55, 139.92, 130.68, 129.06, 128.53, 128.43, 126.63, 125.06, 124.13, 120.15, 120.06, 111.53, 110.38, 104.91, 68.72, 56.07, 53.65, 46.57, 41.98, 29.53, 28.72, 28.06, 26.66, 25.55, 24.12; ESIMS: m/z 498 (M^+).

5.24. 2-[1-(3-[(11a*S*)-7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl]oxypropyl)-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden]malononitrile (11e)

The compound **11e** was prepared according to the method described for the compound **11a**, employing the compound **10e** (627 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11e** as a white solid (312 mg, 62% yield). Mp 90–95 °C; $[\alpha]_D^{27}$ +60.2 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 1H, J = 6.7 Hz), 8.02 (d, 1H, J = 8.2 Hz), 7.72 (t, 1H, J = 7.6 Hz), 7.62 (d, 1H, J = 4.5 Hz), 7.48 (d, 2H, J = 8.4 Hz), 7.36 (t, 1H, J = 6.7 Hz), 6.97 (d, 1H, J = 6.7 Hz), 6.72 (s, 1H), 4.20–4.03 (m, 2H), 3.94 (s, 3H), 3.88–3.77 (m, 1H), 3.71–3.66 (m, 1H), 3.62–3.52 (m, 1H), 2.41–2.23 (m, 4H), 2.09–1.96 (m, 2H), 1.83–1.68 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): 162.30, 150.30, 147.69, 146.52, 140.48, 139.32, 138.98, 130.76, 129.89, 128.56, 126.38, 124.34, 120.23, 115.72, 111.42, 105.07, 67.21, 65.79, 56.02, 53.61, 46.62, 36.96, 29.47, 28.28, 24.06; ESIMS: m/z 503 (M^+).

5.25. 2-[1-(3-[(11*aS*)-7-Methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl]oxybutyl)-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden]malononitrile (11f)

The compound **11f** was prepared according to the method described for the compound **11a**, employing the compound **10f** (641 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11f** as a white solid (346 mg, 67% yield). Mp 92–96 °C; $[\alpha]_D^{27}$ +58.4 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (d, 1H, J = 6.7 Hz), 7.96 (d, 1H, J = 8.1 Hz), 7.70 (t, 1H, J = 5.8 Hz), 7.58 (d, 1H, J = 4.1 Hz), 7.49–7.36 (m, 3H), 6.94 (d, 1H, J = 6.5 Hz), 6.73 (s, 1H), 4.18–4.06 (m, 2H), 4.04 (t, 2H, J = 6.0 Hz), 3.94 (s, 3H), 3.86–3.74 (m, 1H), 3.71–3.66 (m, 1H), 3.63–3.49 (m, 1H), 2.35–2.25 (m, 2H), 2.09–1.90 (m, 4H), 1.64–1.45 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.39, 146.82, 140.46, 130.75, 129.12, 128.73, 128.48, 128.41, 126.62, 125.13, 124.46, 124.12, 120.24, 120.11, 115.51, 111.46, 110.43, 105.26, 105.06, 68.25, 67.29, 55.92, 53.59, 46.57, 38.74, 29.52, 26.18, 25.30, 24.83; ESIMS: m/z 517 (M^+).

5.26. 2-[1-(3-[(11*aS*)-7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl]oxypentyl)-1,2-dihydrobenzo[*c*,*d*]indol-2-yliden]malononitrile (11g)

The compound **11g** was prepared according to the method described for the compound **11a**, employing the compound **10g** (655 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11g** as a white solid (302 mg, 57% yield). Mp 87–92 °C; $[\alpha]_D^{27}$ +56.1 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.01 (d, 1H, J = 6.8 Hz), 7.96 (d, 1H, J = 7.5 Hz), 7.67 (t, 1H, J = 6.7 Hz), 7.58 (d, 1H, J = 3.8 Hz), 7.48–7.40 (m, 3H), 6.88 (d, 1H, J = 6.8 Hz), 6.70 (s, 1H), 4.10–3.96 (m, 2H), 3.94 (t, 2H, J = 6.7 Hz), 3.89 (s, 3H), 3.82–3.74 (m, 1H), 3.70–3.65 (m, 1H), 3.59–3.52 (m, 1H), 2.33–2.28 (m, 2H), 2.08–1.99 (m, 2H), 1.96–1.83 (m, 4H), 1.66–1.58 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.32, 150.75, 147.69, 146.79, 140.53, 139.79, 130.68, 129.14, 128.57, 128.38, 126.62, 125.16, 124.12, 120.17, 120.05, 115.78, 111.48, 110.41, 104.91, 68.62, 68.06, 56.12, 53.59, 46.62, 40.11, 29.56, 28.61, 24.22, 23.41; ESIMS: m/z 532 (M^++1).

5.27. 2-[1-(3-[(11*aS*)-7-Methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl]oxyhexyl)-1,2-dihydrobenzo[*c*,*d*]*indol-2-yliden]malononitrile (*11h)

The compound **11h** was prepared according to the method described for the compound **11a**, employing the compound **10h** (669 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11h** as a white solid (343 mg, 63% yield). Mp 86–91 °C; $[\alpha]_D^{27}$ +54.4 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.02 (d, 1H, J = 6.7 Hz), 7.94 (d, 1H, J = 7.3 Hz), 7.68 (t, 1H, J = 6.7 Hz), 7.57 (d, 1H, J = 3.8 Hz), 7.46–7.42 (m, 3H), 6.89 (d, 1H, J = 6.7 Hz), 6.72 (s, 1H), 4.12–3.97 (m, 2H), 3.94 (t, 2H, J = 6.4 Hz), 3.88 (s, 3H), 3.82–3.76 (m, 1H), 3.70–3.67 (m, 1H), 3.59–3.52 (m, 1H), 2.33–2.24 (m, 2H), 2.09–1.98 (m, 4H), 1.96–1.81 (m, 4H), 1.67–1.52 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.31, 150.49, 147.74, 146.82, 140.56, 140.12, 130.68, 129.00, 128.49, 128.41, 126.63, 125.13, 124.13, 120.51, 120.16, 115.64, 111.56, 110.42, 104.89, 68.72, 67.98, 56.17, 53. 59, 46.57, 41.92, 29.53, 28.62, 28.16, 26.76, 25.35, 24.21; ESIMS: m/z 545 (M^+).

5.28. 2–3-[(7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy]propyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (11i)

The compound **11i** was prepared according to the method described for the compound **11a**, employing the compound **10i** (615 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11i** as a white solid (353 mg, 72% yield). Mp 74–79 °C; $[\alpha]_D^{27}$ +66.0 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.05 (d, 1H, J = 7.5 Hz), 7.95 (d, 1H, J = 6.7 Hz), 7.74 (t, 1H, J = 7.5 Hz), 7.63 (d, 1H, J = 3.7 Hz), 7.55 (s, 1H), 7.46–7.38 (m, 2H), 6.87 (dd, 1H, J = 6.7 Hz), 6.80 (s, 1H), 4.31–4.12 (m, 4H), 4.00 (s, 3H), 3.87–3.78 (m, 1H), 3.74–3.67 (m, 1H), 3.62–3.53 (m, 1H), 2.51–2.42 (m, 2H), 2.34–2.26 (m, 2H), 2.09–2.00 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.78, 150.84, 148.22, 141.07, 137.13, 131.53, 131.03, 130.75, 129.85, 128.35, 120.90, 120.06, 119.68, 118.55, 112.05, 111.29, 103.67, 65.86, 56.55, 54.08, 47.06, 39.12, 29.98, 28.55, 24.55; ESIMS: m/z 492 (M^++1).

5.29. 2–3-[(7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy]butyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (11j)

The compound **11j** was prepared according to the method described for the compound **11a**, employing the compound **10j** (629 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11j** as a white solid (339 mg, 67% yield). Mp 75–80 °C; $[\alpha]_D^{27}$ +38.1 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 1H, J = 7.3 Hz), 7.93 (d, 1H, J = 6.7 Hz), 7.72 (t, 1H, J = 7.6 Hz), 7.64 (d, 1H, J = 3.9 Hz), 7.57(s, 1H), 7.45–7.37 (m, 2H), 6.86 (dd, 1H, J = 6.7 Hz), 6.81 (s, 1H), 4.32–4.14 (m, 4H), 4.02 (s, 3H), 3.86–3.79 (m, 1H), 3.76–3.67 (m, 1H), 3.62–3.53 (m, 1H), 2.52–2.44 (m, 2H), 2.34–2.26 (m, 2H), 2.09–2.02 (m, 4H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.38, 150.66, 147.81, 140.63, 136.56, 131.08, 130.61, 130.37, 129.34, 127.99, 120.32, 119.67, 119.20, 118.13, 111.62, 110.60, 103.03, 68.42, 56.08, 53.66, 46.61, 41.85, 29.58, 26.27, 25.08, 25.20; ESIMS: m/z 506 (M^+).

5.30. 2–3-[(7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy]pentyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (11k)

The compound **11k** was prepared according to the method described for the compound **11a**, employing the compound **10k** (643 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11k** as a white solid (384 mg, 74% yield). Mp 74–80 °C; $[\alpha]_D^{27}$ +34.1 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.08 (d, 1H, J = 8.3 Hz), 7.94 (d, 1H, J = 6.9 Hz), 7.74 (t, 1H, J = 7.7 Hz), 7.67 (d, 1H, J = 4.3 Hz), 7.56–7.43 (m, 3H), 6.79 (s, 1H), 6.73 (d, 1H, J = 7.1 Hz), 4.17–4.02 (m, 2H), 3.93 (s, 3H), 3.88–3.78 (m, 3H), 3.75–3.68 (m, 1H), 3.62–3.53 (m, 1H), 2.36–2.26 (m, 2H), 2.10–1.93 (m, 6H), 1.74–1.62 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.37, 150.71, 147.75, 140.58, 136.52, 131.05, 130.62, 130.34, 129.30, 127.98, 120.13, 119.64, 119.20, 118.01, 111.56, 110.48, 102.88, 68.61, 56.09, 53.64, 46.58, 41.95, 29.55, 28.46, 27.92, 24.12, 23.48; ESIMS: m/z 520 (M^++1).

5.31. 2–3-[(7-Methoxy-5-oxo-2,3,5,11a-tetrahydro-1*H*-benzo[e]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy]hexyl-1,2-dihydro-1λ^6-naphtho[1,8-*cd*]isothiazole-1,1-dione (11l)

The compound **11l** was prepared according to the method described for the compound **11a**, employing the compound **10l** (657 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **11l** as a white solid (357 mg, 67% yield). Mp 75–80 °C; $[\alpha]_D^{27}$ +29.2 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.01 (d, 1H, J = 7.9 Hz), 7.93 (d, 1H, J = 6.9 Hz), 7.72 (t, 1H, J = 6.9 Hz), 7.60 (d, 1H, J = 3.9 Hz), 7.49 (t, 1H, J = 7.9 Hz), 7.44 (s, 1H), 7.38 (d, 1H, J = 8.9 Hz), 6.73 (s, 1H), 6.68 (d, 1H, J = 6.9 Hz), 4.12–3.99 (m, 2H), 3.91 (s, 3H), 3.82–3.77 (m, 3H), 3.70–3.66 (m, 1H), 3.58–3.53 (m, 1H), 2.32–2.25 (m, 2H), 2.06–2.00 (m, 2H), 1.97–1.92 (m, 2H), 1.91–1.84 (m, 2H), 1.59–1.54 (m, 4H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 162.35, 150.74, 147.72, 140.51, 136.54, 131.06, 130.60, 130.32, 129.30, 127.98, 120.01, 119.65, 117.99, 111.44, 110.32, 102.84, 68.70, 56.08, 53.64, 46.59, 41.99, 29.54, 28.70, 28.07, 26.66, 25.51, 24.13; ESIMS: m/z 557 (M^++Na).

6. Cell culture

The anticancer activity of the compounds was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay.[34] Around 1×10^4 cells/well were seeded in 100 μl DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS in each well of 96-well plates and were incubated for 24 h at 37 °C in a CO_2 incubator. After 24 h of incubation, cells were treated with the test compounds for 48 h. After the treatment, 10 μl of MTT (5 mg/mL) was added to each well and the plates were further incubated for 4 h. After incubation, supernatant from each well was carefully removed and formazon crystals were dissolved in 100 μl of dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 540 nm. The MTT assay was performed for all the test compounds in human cancer cells such as A549, A431, Colo-205 and PC-3 and in normal cells (HEK-293).

7. Thermal denaturation studies

The compounds **11a–l** were subjected to DNA thermal melting (denaturation) studies using duplex form calf thymus DNA (CT-DNA) using modification reported procedure.[26] Working solutions were produced by appropriate dilution in aqueous buffer (10 mM NaH_2PO_4/NaH_2PO_4, 1 mM Na_2EDTA, pH 7.00 + 0.01) containing CT-DNA, (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in methanol to obtain a fixed [PBD]/[DNA] molar ratio of 1:5 The DNA-PBD solutions were incubated at 37 °C for 0 h prior to analysis sample were monitored a 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller. Heating was applied at a rate of 1 °C min^{-1} in the 40–90 °C range. DNA helix-coil transition temperatures (T_m) were determined from the maxima in the d (A260)/dT derivative plots. Results for each compound are shown as mean ± standard derivation from the least three determinations and are corrected for the effects of methanol co solvent using a linear correction term. Ligand-induced alteration in DNA melting behavior are given by $\Delta T_m = T_m$ (DNA + PBD) − T_m (DNA alone), where the T_m value for the PBD free CT-DNA is 69.8 ± 0.001 the fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

8. Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases. A549, Lung cancer cells were incubated with compound (**11l**) at 1 μM and 2 μM concentrations for 48 h along with standard DC-81. Untreated and treated cells were harvested, washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide (Sigma Aldrich). Cell cycle was performed by flow cytometry (Becton Dickinson FACS Caliber) as earlier described.[35]

9. Hoechst staining

Cells were seeded at a density of 10,000 cells over 18-mm cover slips and incubated for 24 h. After incubation, cells were treated with the compound **11l** (2 μM) for 24 h along with DC-81 (2 μM). Hoechst 33258 (Sigma Aldrich) was added to the cells at a concentration of 0.5 mg/mL and incubated for 30 min at 37 °C. Later, cells were washed with phosphate buffered saline (PBS). Cells from each cover slip were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic cells based on their relative fluorescence and nuclear fragmentation.[36]

10. Annexin V-FITC

A549 cells (1×10^6) were seeded in six-well plates and incubated for 24 h. Then cells were treated with the compound **11l** at concentrations of 1 μM and 2 μM for 48 h. After 48 h of treatment, cells were harvested by trypsinization and washed with PBS by centrifuging at 3000 rpm for 5 min at 4 °C. Then the cells (1×10^6) were stained with Annexin V-FITC and propidium iodide using the Annexin-V-PI apoptosis detection kit (Invitrogen). Flow cytometry was performed by using FACScan (Becton Dickinson) equipped with a single 488-nm argon laser as described earlier.[37]

11. Caspase 3 activity

Caspase-3 assay was conducted for detection of apoptosis in Lung cancer cell line (A549). The commercially available apoptosis detection kit (Sigma-Caspase 3 Assay kit, Colorimetric) was used. A549 cells treated with compound **11l** for 48 h. After 48 h of treatment, cells were collected by centrifugation, washed once with PBS, and cell pellets were collected. Suspended the cell pellet in lysis buffer and incubated for 15 min. After incubation, cells were centrifuge at 20,000 rpm for 15 min and collected the supernatant. Supernatants were used for measuring caspase 3 activity using an ELISA-based assay, according to the manufacturers instructions.

12. DNA laddering assay

Cells were seeded (1×10^6) in six well plates and incubated for 24 h. After incubation, cells were treated with compound **11l** (1 μM and 2 μM) along with DC-81 (1 μM) for 48 h. Cells were collected and centrifuged at 2500 rpm for 5 min at 4 °C. Pellet was collected, washed with Phosphate buffered saline (PBS). 100 μl of lysis buffer was added to pellet and centrifuged at 3000 rpm for 5 min at 4 °C. To the supernatant, was added 10 μl of 10% SDS and 10 μl of (50 mg/mL) RNase-A and incubated for 2 h at 56 °C. After incubation, added 10 μl (25 mg/mL) of Proteinase K and further incubated for 2 h at 37 °C, then added 65 μl of 10 M Ammonium acetate and 500 μl of ice cold ethanol. Samples were incubated at −80 °C for 1 h. After incubation samples were centri-

fuged at 12000 rpm for 20 min at 4 °C and the pellet was washed with 80% ethanol, air dried for 10 min at room temperature. The pellet was dissolved in 50 μl of TE buffer and DNA laddering assay was performed by using 2% agarose gel electrophoresis.

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