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Original article

Synthesis and biological evaluation of cinnamido linked pyrrolo[2,1-*c*][1,4] benzodiazepines as antimitotic agents

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ABSTRACT

A series of new cinnamido-pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates (**4a**–**d** and **5a**–**d**) and their dimers (**6a**–**d**) have been designed, synthesized and evaluated for their biological activity. The anticancer screening of compound **4a** by the NCI exhibited significant GI50 values ranging from 68 to 732 nM against 53 of 59 human cancer cell lines tested. Compounds **5a**–**d** and **6a**–**d** have also shown remarkable cytotoxic activity with GI50 values <0.1 μM concentrations in a large number of cell lines. Interestingly, compounds **5b** and **6b** have been identified as a new class of inhibitors of tubulin polymerization and their action has been rationalized by the cell cycle arrest in G0 and G2/M phase.

1. Introduction

Cancer remains one of the leading cause of death in the World and as a result there is a pressing need for the development of novel and effective treatments. Cancer cells differ from their normal counterparts in a number of biochemical processes, particularly during the control of cell growth and division. Despite major breakthroughs in many areas of modern medicine over the past 100 years, the successful treatment of cancer remains a significant challenge at the start of the 21st century. Mainly because it is difficult to discover novel agents that selectively kill tumor cells or inhibit their proliferation without the general toxicity, therefore the use of traditional cancer chemotherapy is still very limited. Currently, combination chemotherapy with different mechanisms of action is one of the methods that is being adopted to treat cancer. Therefore, a single molecule containing more than one pharmacophore, each with different mode of action could be beneficial for the treatment of cancer. In recent years there has been growing interest in the design of ligands that could act in a specific manner on more than one target. The development of such hybrid molecules not only lowers the risk of drug–drug interaction in comparison to cocktails but also could enhance the efficacy as well as improve the safety aspects in relation to the drugs that interact on a single target [1–3], and one such recent example is of bleomycin [4].

Microtubules are important in mitosis and cell division; they have been a target for the development of a number of new anticancer drugs [5]. There are two major groups of these antitumor agents, microtubule stabilizers such as paclitaxel [6] and microtubule destabilizers such as colchicine [7], combretastatin A-4 [8], and vinca alkaloids [9]. Combretastatin A-4 (CA-4, Fig. 1), isolated from the bark of the South African tree Combretum caffrum, is one of the well-known natural tubulin binding molecule that affects microtubule dynamics [10]. The spatial relationship between the two aromatic rings of combretastatin A-4 or colchicine and similar other drugs is an important structural feature that determines their ability to bind to tubulin [11]. A number of chalcones have been reported that are similar to CA-4, as active antimitotic agents inhibiting tubulin polymerization [12]. Similarly, there are also several reports on the antiproliferative properties of chalcones substituted with basic groups [13,14]. Xia and coworkers are among the first to demonstrate the improved antiproliferative activity of chalcones that have amino groups [15]. Further, Liu and coworkers have synthesized piperidinyl chalcones as promising antiproliferative agents, many of them have interesting activities (IC50 5 μM) and they also disrupt the cell cycle at G1 and G2/M phases at these concentrations. In contrast, chalcones without the basic substituent have no effect on the cell cycle when tested at their IC50 [16].

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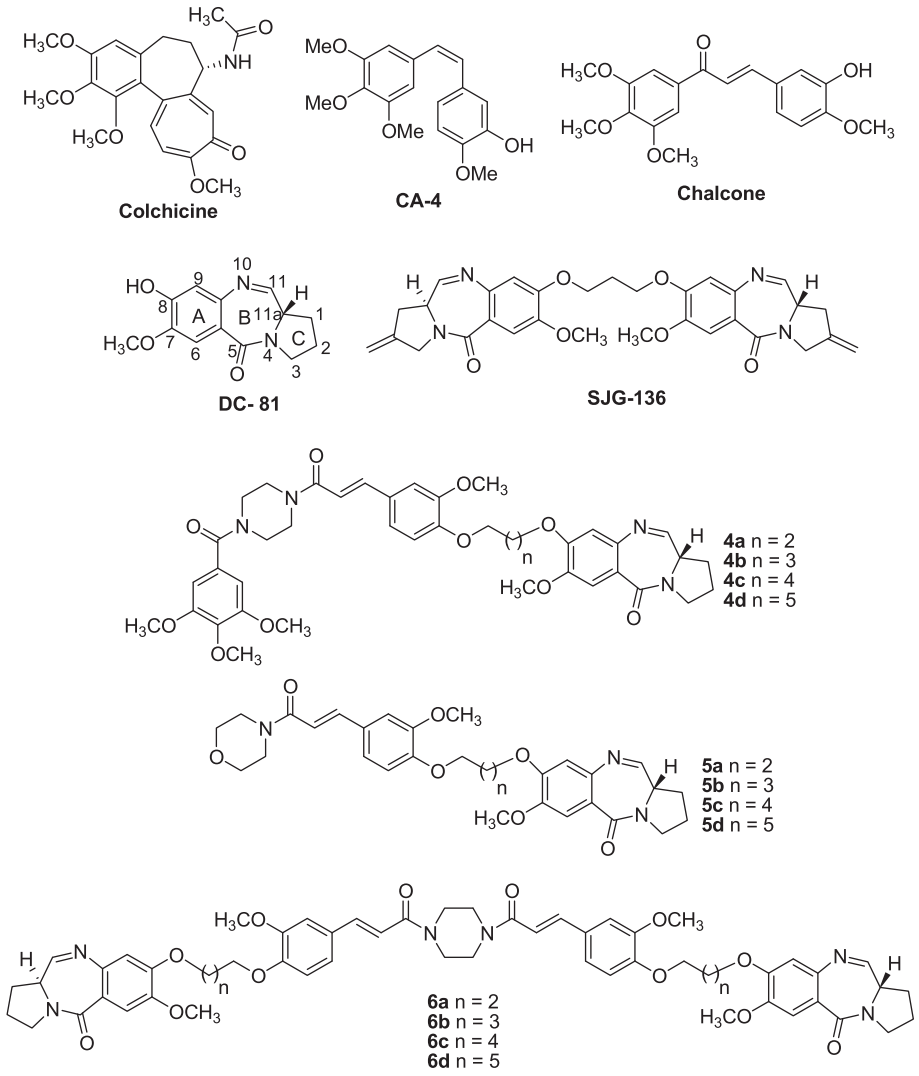
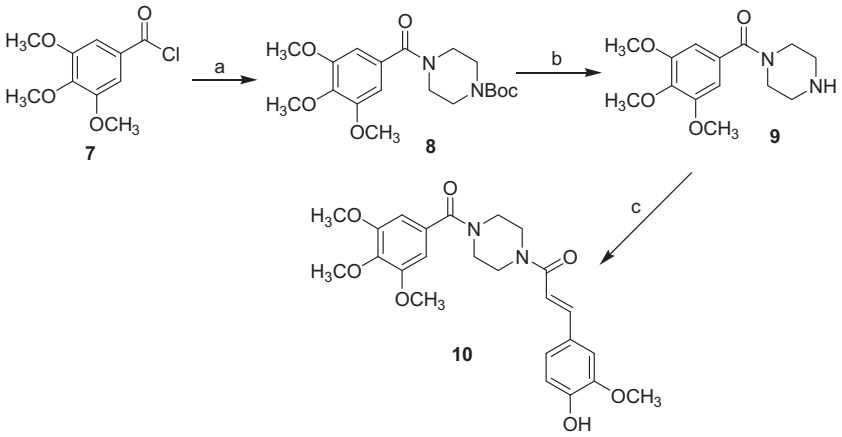


Fig. 1. Chemical structures of tubulin polymerization inhibitors, PBDs, Cinnamido–PBD Conjugates (**4a**–**d**, **5a**–**d** and **6a**–**d**).

Whereas, pyrrolo[2,1-*c*][1,4]benzodiazepines [17] (PBDs), CC-1065 and distamycin exhibit cytotoxic activity that is derived from their ability to bind and monoalkylate DNA in the minor groove [18,19]. Moreover, PBDs are a family of naturally occurring antitumour agents that are known to interact with DNA in a sequences elective manner. PBD monomers such as DC-81 (Fig. 1), anthramycin and tomaymycin are known to monoalkylate DNA by covalently binding to the N_2 of a guanine base in the DNA minor groove through their electrophilic N10–C11 imine (or equivalent carbinolamine) moiety, a process which can block transcription and lead to cytotoxicity [20]. Recently a large number of structurally modified PBDs have been synthesized that have exhibited improved anticancer activity [21–27]. More recently, Hu and coworkers reported that a PBD hybrid (IN6CPBD) can activate the apoptotic pathway mediated by mitochondria and its continuation to the transcription factors like nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [28,29]. The naturally occurring and structurally modified PBDs are known for their potent anticancer activity, however they have been precluded from clinical application due to problems relating to solubility and cardiovascular side-effects [30]. Hence, the development of conjugates or hybrid molecules that contain two types of cytotoxic moieties represent a potential approach in the discovery of new antitumor agents, as they could possess not only high potency but also different binding sites, both the aspects are likely to be useful for the treatment of tumors.

While piperazine is an attractive pharmacological scaffold that is present in many important drugs [31]. It is well known that this heterocyclic backbone could act on various pharmacological targets and that display anticancer [32–39], calcium channel blocking [40–43] and histamine antagonist properties [44,45].

We have been interested in the structural modifications of the PBD ring system [46–49] apart from the development of new synthetic strategies [50–52] for its preparation. Furthermore, modifications of the ethylene bridge in the combretastatin based compounds and changes in chalcones have led to interesting biological properties, particularly cytotoxicity. As mentioned, in view of the importance of the piperazine moiety, it has been considered of interest to incorporate this moiety in such new hybrids of PBD which is also flanked by carbonyl functionalities. Moreover, in another set of series, the aromatic ring of the chalcones has been replaced by a morpholine moiety. This modified cinnamido component has been linked to the PBD ring system through flexible spacers with a view to explore their anticancer activity and to examine their effect on the tubulin polymerization. Further this report investigates the effect of these compounds on cell cycle progression.



Scheme 1. Preparation of Cinnamido promoiety **10**. *Reagents and conditions*: a) TEA, N-Boc piperazine, dry THF, 0 °C to RT, 2 h; b) CF_3COOH, $CHCl_3$, RT, 8 h; c) trans-3-methoxy-4-hydroxy cinnamoyl chloride, TEA, dry THF, 0 °C to RT, 2 h.

2. Results and discussion

2.1. Chemistry

The synthetic pathway (Scheme 1) involves the use of commercially available 3,4,5-trimethoxy benzoic acid as the starting material, which was treated with thionyl chloride and then coupled to N-Boc piperazine in the presence of a base to give the corresponding compound **8**. This upon deprotection of boc by employing TFA in CH2Cl2 gives the compound **9**, which was coupled with trans 4-hydroxy-3-methoxy cinnamic acid chloride provides the required cinnamido promoiety **10**.

Addition of trans 4-hydroxy-3-methoxy cinnamic acid chloride to morpholine in the presence of triethylamine provides compound **11**. Whereas, addition of trans 4-hydroxy-3-methoxy cinnamic acid chloride to piperazine in the presence of base gives a dimer cinnamido promoiety **12** as shown in Scheme 2.

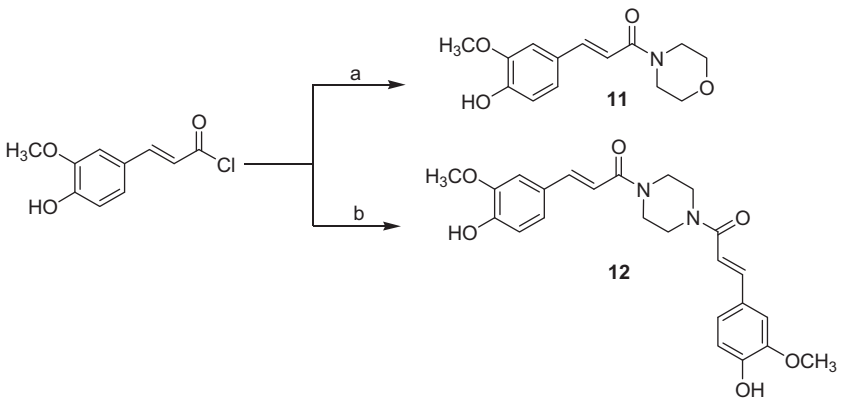
The synthesis of cinnamido-pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) conjugates (**4**, **5**) and their dimers (**6**) was carried out starting from the (2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethylthioacetal (**13**), which was prepared by literature method [53] and this upon debenzylation provides (2*S*)-*N*-(4-hydroxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethylthioacetal precursor (**14**). Etherification of this hydroxyl compound **14** with dibromopropane in the presence of K_2CO_3 in acetone affords (2*S*)-*N*-[4-(3-bromopropoxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15a**. Further coupling of this with the cinnamido promoieties **10**, **11** and **12** respectively in the presence of K_2CO_3 in acetonitrile produce corresponding nitro compounds **16**, **17** and **20**. Further these upon reduction with $SnCl_2 \cdot 2H_2O$ provide the aminothioacetal intermediates (**18**, **19** and **21**). Finally deprotection of these aminothioacetals by employing $HgCl_2/CaCO_3$ affords the target imine containing PBD conjugates (**4**, **5** and **6**) as shown in Schemes 3 and 4.

2.2. Evaluation of biological activity

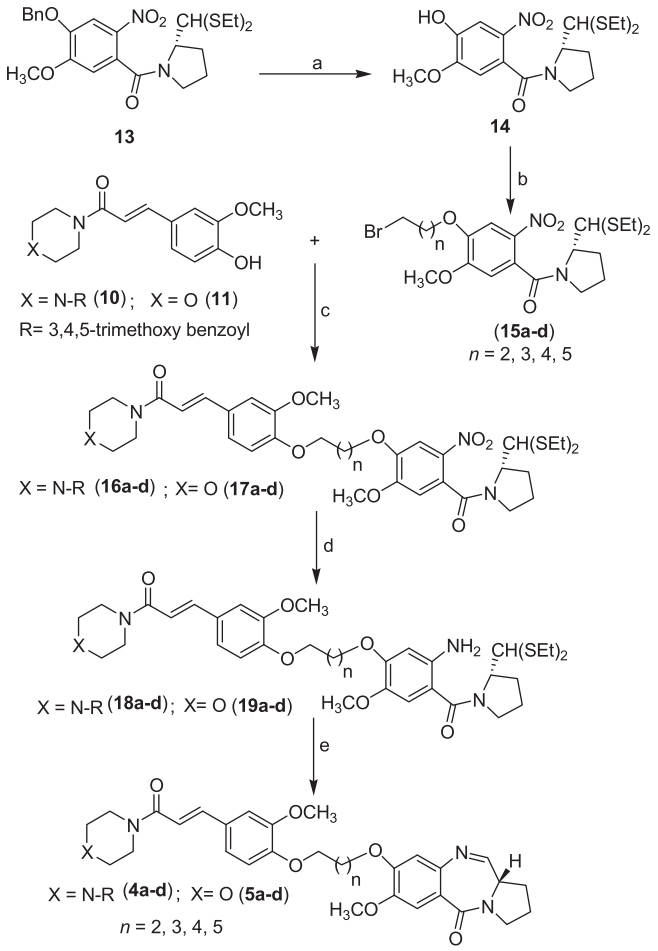
2.2.1. Anticancer activity

Compound **4a** has been evaluated for the anticancer activity against a panel of 60 human cancer cell lines of the National Cancer Institute (NCI), Bathesda and the results are shown in Table 1. Compound **4a** is remarkably potent with GI50 values ranging from 68 to 732 nM towards 53 of the 59 human cancer cell lines tested. This compound is active between 73 and 695 nM in all leukemia, CNS, prostrate and melanoma cancer cell lines. Interestingly it is highly active against some specific cell lines like CCRF-CEM (leukemia), NCI-H522 (non-small cell lung cancer), and MCF7, MDA-MB-468 (breast cancer). The mean graph midpoint values of compound **4a** are log10 GI50 (−6.48), log10 TGI (−5.4) and log10 LC_{50} (−4.43) and these results indicate that **4a** possesses potent broad-spectrum anticancer activity.

The promising activity of compound **4a** prompted us to evaluate the anticancer activity of other related compounds **4b**–**d**, **5a**–**d** and **6a**–**d** in selected human cancer cell lines like A-549, Hop62 (lung);



Scheme 2. Preparation of Cinnamido promoieties**11** and **12**. *Reagents and conditions*: a) dry THF, morpholine, TEA, 0 °C to RT, 2 h; b) dry THF, piperazine, TEA, 0 °C to RT, 2 h.



Scheme 3. Preparation of Cinnamido–PBD conjugates **4a–d** and **5a–d**. *Reagents and conditions*: a) EtSH, $BF_3 \cdot OEt_2$, CH_2Cl_2, 12 h; b) dibromoalkanes, K_2CO_3, acetone, reflux, 48 h; (c) K_2CO_3, acetonitrile, 18 h, reflux, 65–73%; (d) $SnCl_2 \cdot 2H_2O$, MeOH, reflux, 4 h; e) $HgCl_2$, $CaCO_3$, $CH_3CN \cdot H_2O$ (4:1), RT, 12 h 60–65%.

KB, GURAV, DWD (oral); SiHa (cervix); MCF7 and ZR75-1 (breast); Colon205 (colon); PC3 (prostate) and A2780 (ovarian) by employing the sulforhodamine B (SRB) assay. The results described in Table 2 show that all the compounds are significantly cytotoxic with the concentration of the drug that produced 50% inhibition of cell growth (GI50) ranging from <0.1 to 2.5 μM. Among all the compounds synthesized, compounds **5c**, **5d**, **6b** and **6d** displayed significant cytotoxicity with a GI50 value <0.1 μM against six cell lines of the eleven cell lines examined. Similarly, compounds **5a** and **6a** (in five cell lines), **6c** (in four cell lines), **4b** and **4c** (in two cell lines) and **5b** (in one cell line) have shown promising anticancer activity with a GI50 value <0.1 μM.

The anticancer activity of these new PBD conjugates has been compared to DC-81 (PBD monomer) and it is interesting to observe that all these conjugates possess better activity profile than DC-81 as shown in Table 2. The cinnamido moieties (**10** and **11**) have also been evaluated for their anticancer potential and it is observed that they do not exhibit any significant activity (GI50 > 10^{-4}). These results clearly indicate that the hybrid compounds are more effective than their individual moieties towards their anticancer potential and also emphasized that linking/combination approach certainly has benefits in designing in superior anticancer compounds.

2.2.2. Inhibition of tubulin polymerization

Since these ten new conjugates possess a chalcone subunit, it has been considered of interest to investigate their effect on tubulin polymerization. One of the possible explanations of compounds showing anticancer activity and cell cycle arrest is the inhibition of tubulin polymerization to functional microtubules as it is observed with antimitotic agents such as nocodazole and cholchicine. As tubulin subunits heterodimerize and self-assemble to form microtubules in a time dependent manner, we have investigated the progression of tubulin polymerization by monitoring the absorbance at 340 nm in 384 well plate for 1 h at 37 °C with and without the compounds at 2.5 μM (final concentration) concentration solubilized in DMSO. Amongst the ten molecules examined, **5b** and **6b** inhibited tubulin polymerization to nearly 40% compared to controls and a similar pattern of inhibition has been observed with the positive control, nocodozole (Fig. 2). The compounds **5b** and **6b** also demonstrated a dose dependent inhibition with IC50 values of ~16.4 μM and ~27.9 μM respectively.

2.2.3. Immunohistochemistry of tubulin

In order to substantiate the observed in vitro effects of the compounds on the inhibition of tubulin polymerization to functional microtubules, immunohistochemistry studies have been carried out to examine the in situ effects of compounds **5b** and **6b** on cellular microtubules and their special arrangement. In this study, untreated human breast cancer cells (MCF-7) displayed the normal distribution of microtubules (Fig. 3a). However, cells treated with 2.5 μM concentration of compounds **5b**, **6b** and nocodazole demonstrated disrupted microtubule organization as seen in Fig. 3b–d, thus exemplifying the inhibition of tubulin polymerization.

2.2.4. Cell cycle analysis and apoptosis (flow cytometry)

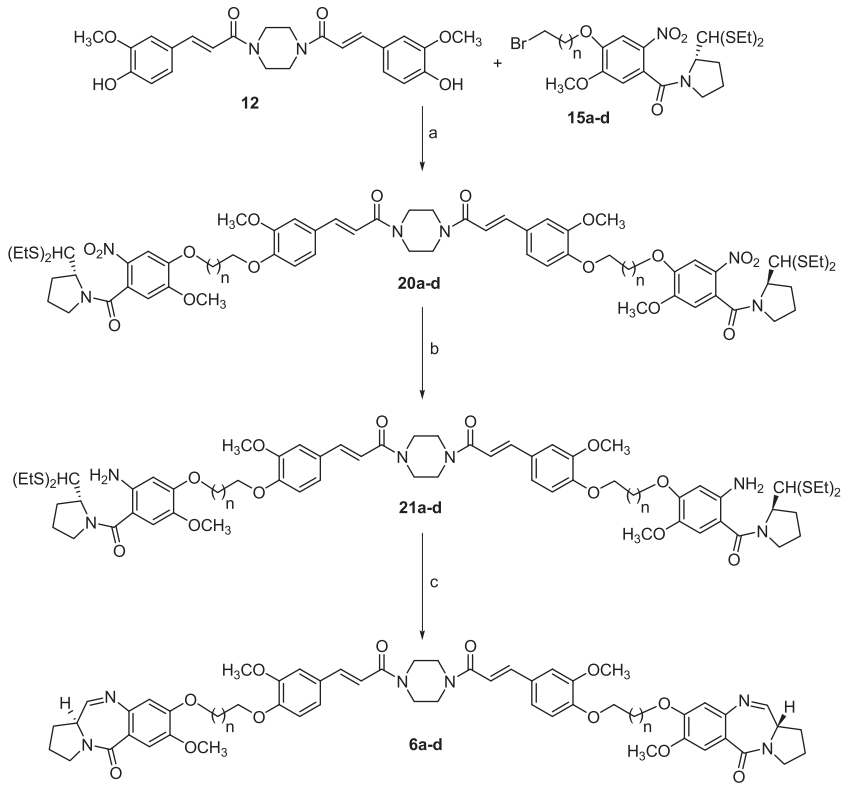
Since the compounds **5b** and **6b** mediate their cytotoxic effects by inhibiting the polymerization of tubulin, we have examined whether the same could be reflected in the pattern of cell cycle arrest. Towards this, the cell cycle dependent DNA content was determined by flow cytometry using propidium iodide staining according to the standard protocol. The effect of compounds **5b** and **6b** (2 μM) along with positive controls i.e., nocodazole and podophyllotoxin (1 μM) were examined in MCF-7 breast cancer cells treated individually with these compounds for 48 h. The results demonstrate that there is an increase in G0 and G2/M phase in cells treated with compounds **5b** and **6b** compared to the controls. Although the effect of **5b** and **6b** at G0 phase is comparable to that of nocodazole and podophyllotoxin (Figs. 4 and 5), they have demonstrated a less pronounced effect at G2/M phase possibly due to the presence of two distinctively different functional moieties, ie., PBD and chalcones. Nevertheless, these results indicate that the induction of cellular apoptosis by **5b** and **6b** could be by virtue of their inhibitory effects on tubulin polymerization as there is an increase in cell cycle arrest at metaphase with parallel increase in apoptotic cells at G0 phase.

2.2.5. Effect of *5b* and *6b* on nuclear condensation

Since several of the microtubule disrupting agents are also known to induce apoptosis in cells. We have analyzed the cellular effects of compounds **5b** and **6b** on nuclear condensation by Hoechst staining. The results indicate that compared to controls, cells treated with compounds **5b** and **6b** that show a dose dependent effect on nuclear condensation between 1 and 3 μM (Fig. 6). However, significant nuclear condensation is observed with nocodazole and podophyllotoxin even at 2 μM concentration (Fig. 6). These results clearly demonstrate that compounds **5b** and **6b** are effective in inducing cellular apoptosis.

3. Conclusion

In conclusion, we have designed and synthesized new cinnamido- pyrrolobenzodiazepine conjugates and their dimers. These



Scheme 4. Preparation of Cinnamido–PBD dimers **6a**–**d**. *Reagents and conditions*: a) K_2CO_3, acetonitrile, reflux, 18 h; 63–75% b) $SnCl_2 \cdot 2H_2O$, MeOH, reflux, 4 h; c) $HgCl_2$, $CaCO_3$, $CH_3CN \cdot H_2O$ (4:1), RT, 12 h 60–63%.

compounds exhibit significant anticancer activity with GI50 values ranging from nanomolar to micromolar concentrations. Among these compounds **5b** and **6b** have shown potent antitubulin activity in vitro as well as disruption of microtubule organization within cells. Further, cell cycle analysis indicated that these compounds induced cell cycle arrest at metaphase with a concomitant increase in apoptotic cells. The compounds **5b** and **6b** have also shown a significant increase in apoptotic cells as observed by DNA condensation and fragmented nuclei. Cytotoxic assays employing individual moieties present in **5b** and **6b** along with DC-81 clearly indicate that the hybrid molecules (**5b** and **6b**) are more potent than their individual moieties. These results provide an insight for future direction in the development of such conjugates.

4. Experimental protocols

Reaction progress was monitored by thin-layer chromatography (TLC) using GF254 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 hydride). 1H 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 cinnamido-PBD conjugates

4.1.1. tert-Butyl-4-(3,4,5-trimethoxybenzoyl)-1-piperazinecarboxylate (*8*)

To a stirred solution of N-boc piperazine (372 mg, 1.0 mmol) in dry THF was added triethylamine (1.1 mL, 4.0 mmol) followed by 3,4,5-trimethoxy benzoylchloride (458 mg, 1.0 mmol) at 0 °C. The reaction mixture was stirred for 2 h and the reaction was monitored by TLC. After completion of the reaction, THF was evaporated under vacuum to get the crude product. This was further purified by column chromatography (30% EtOAc–Hexane) to afford the pure compound **8** (Yield 646 mg, 85%). $R_f = 0.58$ (EtOAc–Hexane 2:8); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.59 (s, 2H, Ar**H**), 3.88 (s, 6H, OC**H3**), 3.84 (s, 3H, OC**H3**), 3.44–3.66 (m, 8H, N–C**H2**–), 1.47 ppm (s, 9H, 3C**H3**); ESI-MS: *m*/*z* 382 $[M + 1]^+$

4.1.2. Piperazino(3,4,5-trimethoxyphenyl)methanone (*9*)

To a solution of boc-protected compound **8** (660 mg, 1 mmol) in dry dichloromethane was added trifluoroacetic acid (1.73 mL) at 0 °C and stirred under nitrogen for 12 h, the reaction mixture was concentrated under vacuum to afford compound **9** and then it was used directly in the next step.

4.1.3. (E)-3-(4-Hydroxy-3-methoxyphenyl)-1-[4-(3,4,5-trimethoxybenzoyl)piperazino]-2-propen-1-one (*10*)

To a stirred solution of piperazino(3,4,5-trimethoxyphenyl) methanone (840 mg, 1.0 mmol) in dry THF was added triethylamine (1.5 mL) followed by 4-hydroxy-3-methoxy cinnamic acid chloride in dry THF (636 mg, 1.0 mmol) at 0 °C. The reaction mixture was stirred for 2 h and the reaction was monitored by TLC. After completion of the reaction, THF was evaporated under vacuum to get the crude product. This was further purified by column chromatography (98:2 $CHCl_3$–MeOH) to afford the pure compound **10**.

Table 1
In vitro cytotoxicity of the compound **4a** on selected human cancer cell lines.[a]

Cancer panel/cell line	GI50 (μM) **4a**	Cancer panel/cell line	GI50 (μM) **4a**
Leukemia		*Renal*	
CCRF-CEM	0.08	786-0	0.27
HL-60(TB)	0.18	A498	0.19
K-562	0.69	ACHN	2.54
MOLT-4	0.23	CAKI-1	2.11
SR	0.28	RXF 393	0.54
		SN12C	0.27
		TK-10	0.29
		UO-31	2.59
Non-small cell lung		*Prostate*	
A549/ATCC	1.89	PC-3	0.73
EKVX	0.72	DU-145	0.33
HOP-62	0.23		
HOP-92	0.49	*Ovarian*	
NCI-H226	0.31	IGROV1	0.24
NCI-H23	0.26	OVCAR-3	0.21
NCI-H322M	0.34	OVCAR-4	1.07
NCI-H460	0.24	OVCAR-5	0.29
NCI-H522	0.06	OVCAR-8	0.45
		SK-OV-3	0.61
Colon		*Breast*	
COLO 205	0.19	MCF7	0.08
HCC-2998	0.24	NCI/ADR-RES	
HCT-116	0.17	MDA-MB-	8.91
HCT-15	2.98	231/ATCC	0.23
HT29	0.26	HS 578T	0.23
KM12	0.21	MDA-MB-435	0.18
SW-620	0.17	BT-549	0.15
		T-47D	0.23
		MDA-MB-468	0.07
CNS		*Melanoma*	
SF-268	0.24	LOX IMVI	0.24
SF-295	0.31	MALME-3M	0.17
SF-539	0.24	M14	0.32
SNB-19	0.38	SK-MEL-2	0.26
SNB-75	0.21	SK-MEL-28	0.20
U251	0.18	SK-MEL-5	0.19
		UACC-257	0.35
		UACC-62	0.16

[a] Data obtained from NCI's in vitro anticancer activity cells screen.

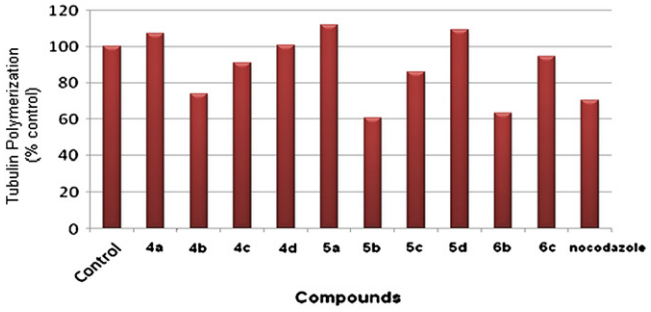


Fig. 2. Effect of compounds on tubulin polymerization: tubulin polymerization assay was carried out in a reaction mixture that contained PEM buffer, GTP (1 mM) and in the presence or absence of test compounds at 2.5 μM concentration. The reaction was initiated by the addition of GTP to all the wells. Tubulin polymerization was monitored by the increase in absorbance at 340 nm using Dynex multimode plate reader at 37 °C. Absorbance was recorded at every 2 min intervals for up to 1 h. Nocodazole was used as positive control. Data shown are the representative of three separate experiments.

(Yield 1.14 g, 84%): R_f = 0.62 (EtOAc–Hexane 8:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.54 (d, 1H, J = 15.10 Hz, olefinic-**H**), 7.02 (d, 1H, J = 7.55 Hz, Ar**H**), 6.90 (s, 1H, Ar**H**), 6.81 (d, 1H, J = 8.30 Hz, Ar**H**), 6.53–6.64 (m, 3H Ar**H** and Olefinic-**H**), 5.86–5.99 (s, H, **OH**), 3.87 (s, 3H, OC**H3**); 3.81 (s, 6H, OC**H3**), 3.79 (s, 3H, OC**H3**), 3.53–3.74 ppm (m, 8H, N–C**H2**–). ESI-MS: m/z 457 $[M + 1]^+$

4.1.4. (E)-3-(4-Hydroxy-3-methoxyphenyl)-1-morpholino-2-propen-1-one (**11**)

To a stirred solution of morpholine (372 mg, 1.0 mmol) in dry THF was added triethylamine (1.1 mL, 4.0 mmol) followed by 4-hydroxy-3-methoxy cinnamic acid chloride in dry THF (458 mg, 1.0 mmol) at 0 °C. The reaction mixture was stirred for 2 h and the reaction was monitored by TLC. After completion of the reaction, THF was evaporated under vacuum to get the crude product. This was further purified by column chromatography (70% EtOAc–Hexane) to afford the pure compound **11** (Yield 646 mg, 85%): R_f = 0.59 (EtOAc–Hexane 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.64 (d, 1H, J = 15.42 Hz, olefinic-**H**); 7.08 (d, 1H, J = 8.08 Hz, Ar**H**); 6.99 (s, 1H, Ar**H**); 6.89 (d, 1H, J = 8.08 Hz, Ar**H**); 6.68 (d, 1H, J = 14.69 Hz, olefinic-**H**); 6.03 (s, 1H, O**H**); 3.93 (s, 3H, OC**H3**); 3.62–3.72 ppm (m, 8H, morpholine-**H**). ESI-MS: m/z 264 $[M^+]$

Table 2
GI_{50} values (in μM) for compounds **4b**–**d**, **5a**–**d** & **6a**–**d** in selected human cancer cell lines.[a]

Compd	ZR-75[b]	A-549[c]	A2870[d]	Hop62[c]	KB[e]	SiHa[f]	Gurav[c]	MCF7[b]	Colo205[g]	DWD[e]	PC3[h]
4b	0.15	0.12	0.15	0.17	0.15	0.17	0.11	0.14	<0.1	0.11	<0.1
4c	0.14	0.11	0.11	0.15	0.12	0.14	0.13	0.13	<0.1	<0.1	0.14
4d	0.21	0.16	0.18	2.6	1.9	2.5	1.9	0.18	2.1	0.17	1.9
5a	0.12	<0.1	<0.1	0.14	<0.1	0.12	0.10	<0.1	0.10	<0.1	0.13
5b	0.15	0.11	0.12	0.16	0.13	0.14	0.13	0.14	0.12	<0.1	0.14
5c	<0.1	<0.1	0.14	0.17	0.15	0.17	0.16	<0.1	<0.1	<0.1	<0.1
5d	0.14	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.15	0.15	0.15	0.17
6a	<0.1	<0.1	0.12	0.15	0.14	0.16	0.14	<0.1	<0.1	<0.1	0.15
6b	0.15	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.15	0.15	0.12	0.16
6c	<0.1	0.11	0.16	0.18	2.0	2.0	2.3	1.0	<0.1	<0.1	<0.1
6d	0.21	0.15	<0.1	<0.1	<0.1	<0.1	<0.1	1.9	<0.1	<0.1	0.15
DC-81	2.37	0.16	0.14	0.15	0.17	0.17	0.16	0.17	0.11	1.49	0.20
ADR	0.12	<0.1	<0.1	0.12	0.13	0.15	0.14	<0.1	0.13	0.12	0.16

[a] 50% Growth inhibition and the values are mean of three determinations.
[b] Breast cancer.
[c] Lung cancer.
[d] Ovarian cancer.
[e] Oral cancer.
[f] Cervix cancer.
[g] Colon cancer.
[h] Prostate cancer.
ADR is adriamycin.

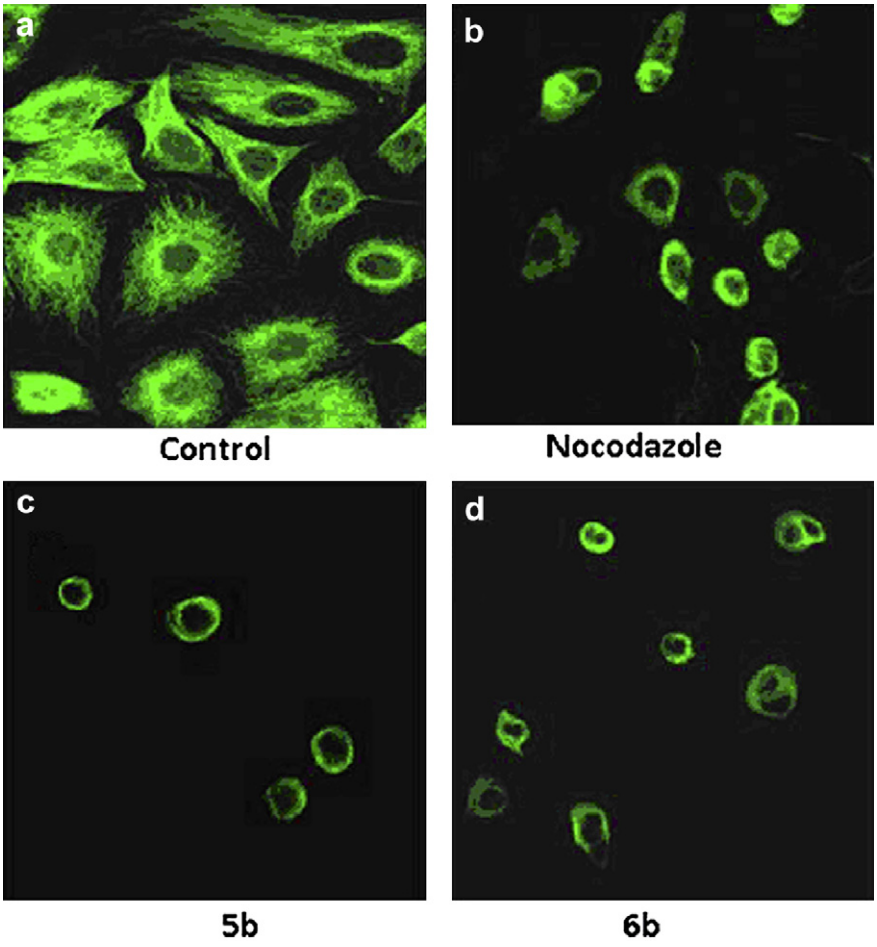


Fig. 3. Immunohistochemistry analysis of microtubules: MCF-7 cells were grown on glass cover slips; incubated for 48 h with Nocodazole (b) **5b** (c) and **6b** (d) at 2.5 μM concentration, untreated cells were considered as controls (a). Following the termination of incubation, cells were fixed and immunohistochemistry was performed as described in the "Experimental Section" employing anti tubulin (mouse monoclonal) antibody followed by FITC conjugated secondary antibody. Photographs were taken using Olympus confocal microscope. Data is the representative of pictures.

*4.1.5. (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one **(12)***

To a stirred solution of piperazine (200 mg, 1.0 mmol) in dry THF was added triethylamine (1.1 mL, 4.0 mmol) followed by 4-hydroxy-3-methoxy cinnamic acid chloride in dry THF (1.06 g, 2.0 mmol) at 0 °C. The reaction mixture was stirred for 2 h and the reaction was monitored by TLC. After completion of the reaction, THF was evaporated under vacuum to get the crude product. This was further purified by column chromatography (100% EtOAc) to afford the pure compound (Yield 1.64 g, 75%): $R_f = 0.59$ (EtOAc); mp 152–154 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.59 (d, 2H, $J = 14.69$ Hz, olefinic-**H**), 7.08 (d, 2H, $J = 10.28$ Hz, Ar**H**), 6.92 (d, 2H, $J = 12.48$ Hz, Ar**H**), 6.85 (s, 2H, Ar**H**), 6.64 (d, 2H, $J = 15.42$ Hz, olefinic-**H**), 5.76 (brs, 2H, O**H**), 3.95 (s, 6H, OC**H3**), 3.42–3.70, ppm (m, 8H, NC**H2**). ESI-MS: m/z 439 [M^+]

*4.1.6. (2S)-N-(4-Hydroxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethylthioacetal (**14**)*

To a stirred solution of EtSH (1.91 g, 19.0 mmol) and BF3.OEt2 (1.41 g, 10 mmol) in dichloromethane was added drop wise to the solution of the compound **13** (0.490 g, 1 mmol) in dichloromethane (10 mL) at room temperature. Stirring was continued until TLC indicated completion of the reaction. The solvent was evaporated under vacuum. The residue, thus obtained was quenched with bicarbonate solution (2 × 25 mL) and then extracted with ethyl acetate (3 × 25 mL). The combined organic phases were washed with saturated brine (1 × 25 mL), dried over Na2SO4 and the solvent removed under vacuum to afford the crude product. This was further purified by column chromatography using EtOAc–hexane (7:3) as eluent to afford compound **14** (yield 300 mg, 75%): $R_f = 0.59$ (EtOAc–hexane 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.6 (s, 1H, Ar**H**), 6.75 (s, 1H, Ar**H**), 4.85 (d, 1H, $J = 7.0$ Hz, –**CH**–), 4.60–4.70 (m, 1H, –**CH**–), 3.95 (s, 3H, OC**H3**), 3.20–3.32 (m, 2H, NC**H2**–), 2.70–2.88 (m, 4H, –**CH2**–), 1.75–2.35 (m, 4H, –**CH2**–), 1.20–1.40 (m, 6H –C**H3**). EI-MS: m/z 400 [M^+]

*4.1.7. (2S)-N-[4-(3-Bromopropoxy-5-methoxy-2-nitrobenzoyl)] pyrrolidine-2-carboxaldehyde diethylthioacetal **(15a)***

To a solution of compound **14** (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 (2:8). 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 Na2SO4 and evaporated under vacuum. The residue, thus obtained was purified by column chromatography using EtOAc–hexane (2:8) to afford compound **15a** as yellow liquid (Yield 440 mg, 96%): $R_f = 0.61$ (EtOAc–hexane 2:8); ^{1}H NMR (300 MHz,

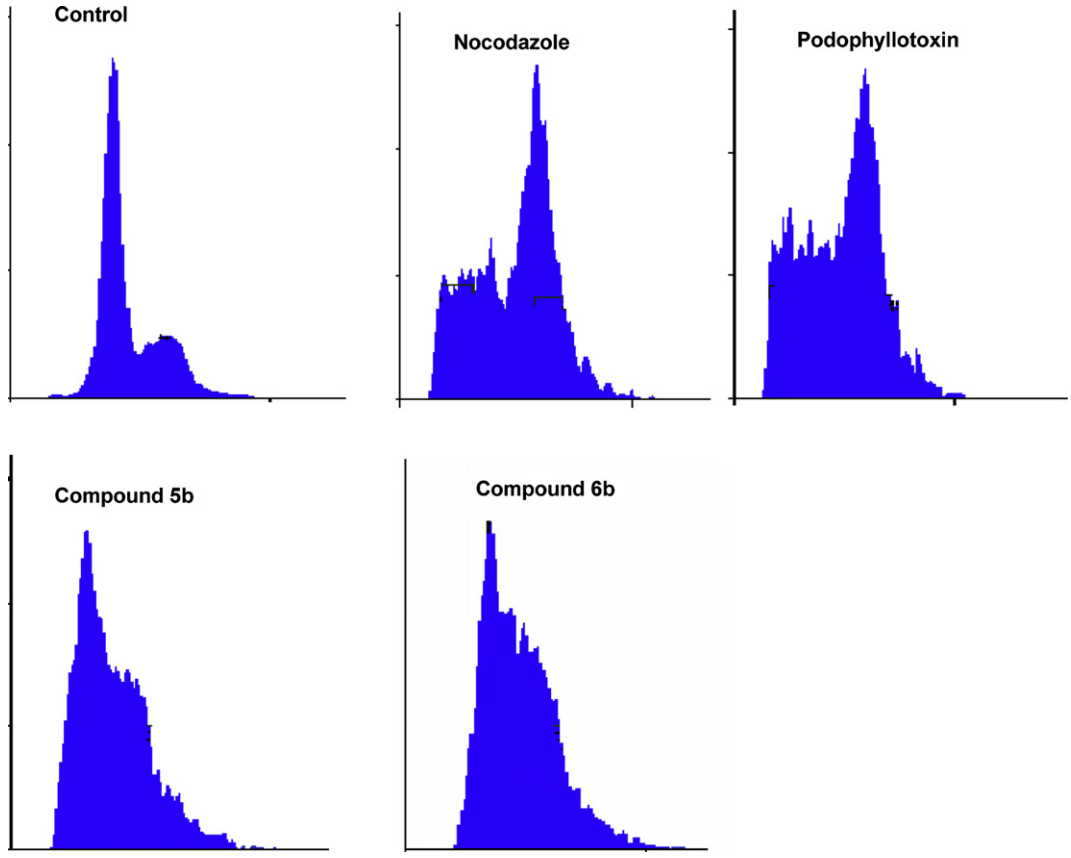


Fig. 4. Effect on Cell cycle and apoptosis: (A) MCF-7 breast cancer cells. In 6 well plates were treated with compounds **5b** (2 μM), **6b** (2 μM), nocodozole (1 μM) and podophyllotoxin (1 μM) for 48 h. Cells were trypsinized, washed with PBS and were fixed in 70% ethanol. Before analysis, cells were finely dispersed and stained with propidium iodide as described in the "Experimental Section". Stained cells were analyzed by flow cytometry. (B) The percentage of cells treated with compounds **5b**, **6b**, nocodozole and podophyllotoxin at different phases of cell cycle.

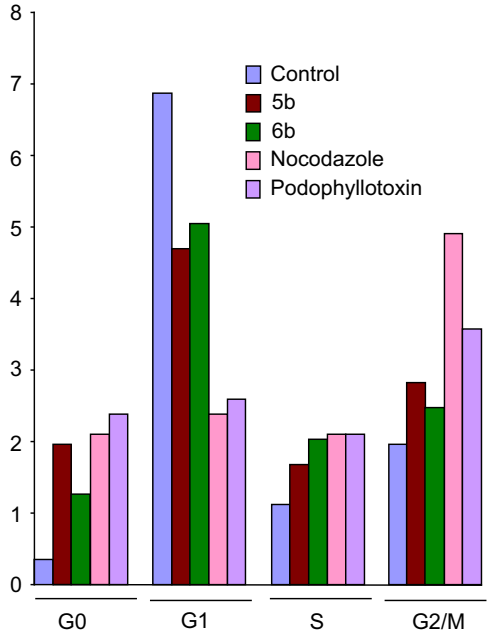


Fig. 5. Effect of nocodozole, podophyllotoxin and the compounds **5b** and **6b** on distribution of cells at various stages of cell cycle.

$CDCl_3$): δ 7.65 (s, 1H, Ar**H**), 6.80 (s, 1H, Ar**H**), 4.82–4.87 (d, 1H, J = 4.3 Hz, −C**H**−), 4.60–4.71 (m, 1H, −C**H**−), 3.98–4.10 (t, 2H, J = 6.0 Hz, OC**H2**), 3.95 (s, 3H, OC**H3**), 3.52–3.59 (t, 2H, J = 6.2 Hz, −C**H2**), 3.15–3.30 (m, 2H, −C**H2**−), 2.60–2.90 (m, 4H, −C**H2**−), 1.70–2.40 (m, 6H, −C**H2**−), 1.21–1.45 ppm (m, 6H, −C**H3**). ESI-MS: m/z 543 [M + Na]

*4.1.8. (2S)-N-[4-(4-Bromobutoxy-5-methoxy-2-nitrobenzoyl)] pyrrolidine-2-carboxaldehyde diethylthioacetal **(15b)***

The compound **15b** was prepared following the method described for the compound **15a**, employing 1,4-dibromobutane (226 mg, 1.2 mmol) and anhydrous K_2CO_3 (483 mg, 4 mmol) and the crude product was purified by column chromatography to afford the compound **15b** as yellow liquid (Yield 421 mg, 90%): R_f = 0.55 (EtOAc–hexane 2:8); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.71 (s, 1H, Ar**H**), 6.92 (s, 1H, Ar**H**), 4.79–4.80 (d, 1H, J = 4.3 Hz, −C**H**−), 4.61–4.70 (m, 1H, −C**H**−), 3.98–4.10 (t, 2H, J = 6.0 Hz, OC**H2**), 3.95 (s, 3H, OC**H3**), 3.55–3.60 (t, 2H, J = 6.2 Hz, −C**H2**), 3.17–3.32 (m, 2H, −C**H2**−), 2.60–2.90 (m, 4H, −C**H2**−), 1.74–2.48 (m, 8H, −C**H2**−), 1.19–1.43 ppm (m, 6H, −C**H3**). ESI-MS: m/z 557 [M + Na]

*4.1.9. (2S)-N-[4-(5-Bromopentoxy-5-methoxy-2-nitrobenzoyl)] pyrrolidine-2-carboxaldehyde diethylthioacetal **(15c)***

The compound **15c** was prepared following the method described for the compound **15a**, employing 1,5-dibromopentane (282 mg, 1.2 mmol) and anhydrous K_2CO_3 (565 mg, 4 mmol) and the crude product was purified by column chromatography to afford the compound **15c** as yellow liquid (Yield 512 mg, 91%): R_f = 0.59 (EtOAc–hexane 2:8); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (s, 1H, Ar**H**),

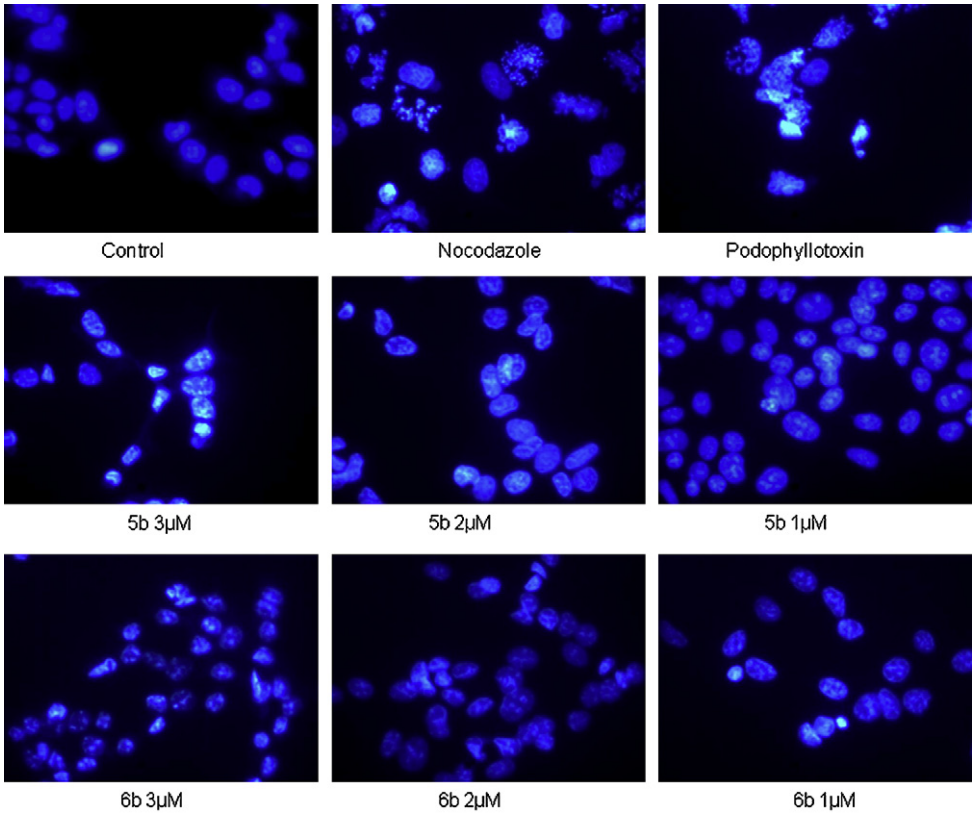


Fig. 6. Analysis of nuclear morphology: MCF-7 cells were grown on cover slips in 6 well plates were treated with compounds **5b** (1–3 μM), **6b** (1–3 μM), nocodozole (1 μM) and podophyllotoxin (1 μM) for 48 h. Following the termination of incubation, cells were incubated with Hoechst (5 μM) for 30 min. Later, cells were washed thrice with PBS and photographs were taken using Olympus fluorescence microscope equipped with DAPI filter settings. Data is the representative of five different fields of view.

6.97 (s, 1H, Ar**H**), 4.77–4.82 (d, 1H, $J = 4.3$ Hz, –C**H**–), 4.59–4.69 (m, 1H, –C**H**–), 3.96–4.09 (t, 2H, $J = 6.0$ Hz, OC**H2**), 3.94 (s, 3H, OC**H3**), 3.52–3.60 (t, 2H, $J = 6.2$ Hz, –C**H2**), 3.18–3.33 (m, 2H, –C**H2**–), 2.62–2.98 (m, 4H, –C**H2**–), 1.73–2.49 (m, 8H, –C**H2**– and –C**H3**). ESI-MS: m/z 571 [M + Na]

*4.1.10. (2S)-N-[4-(6-Bromohexyloxy-5-methoxy-2-nitrobenzoyl)] pyrrolidine-2-carboxalde-hyde diethylthioacetal **(15d)***

The compound **15d** was prepared following the method described for the compound **15a**, employing 1,6-dibromohexane (285 mg, 1.2 mmol) and anhydrous K_2CO_3 (507 mg, 4 mmol) and the crude product was purified by column chromatography to afford the compound **15d** as yellow liquid (Yield 512 mg, 93%): $R_f = 0.60$ (EtOAc–hexane 2:8); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.69 (s, 1H, Ar**H**), 6.94 (s, 1H, Ar**H**), 4.71–4.82 (d, 1H, $J = 4.3$ Hz, –C**H**–), 4.51–4.67 (m, 1H, –C**H**–), 3.98–4.11 (t, 2H, $J = 6.0$ Hz, OC**H2**), 3.93 (s, 3H, OC**H3**), 3.54–3.62 (t, 2H, $J = 6.2$ Hz, –C**H2**), 3.14–3.31 (m, 2H, –C**H2**–), 2.60–2.97 (m, 4H, –C**H2**–), 1.75–2.50 (m, 8H, –C**H2**–), 1.19–1.50 ppm (m, 10H, –C**H2**– and –C**H3**). ESI-MS: m/z 585 [M + Na]

*4.1.11. (2S)-N-{4-[3-[2-Methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]propoxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (**16a**)*

To a stirred solution of (*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-[4-(3,4,5-trimethoxy benzoyl)piperazino]-2-propen-1-one **10** (456 mg, 1 mmol) in dry acetonitrile (20 mL) was added anhydrous K_2CO_3 (544 mg, 5.0 mmol) and compound (2*S*)-*N*-[4-(3-bromopropoxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15a** (625 mg, 1.2 mmol). The reaction mixture was stirred at reflux temperature for 12 h and the reaction was monitored by TLC. After completion of the reaction, K_2CO_3 was removed by suction filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography (98:2 $CHCl_3$–MeOH) to afford the pure compound **16a** (Yield 672 mg, 75%): $R_f = 0.61$ (MeOH–$CHCl_3$ 2:98); Light yellow solid. mp 130–132 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.66 (d, 1H, $J = 14.95$ Hz, olefinic-**H**); 7.50 (s, 2H, Ar**H**); 7.02 (s, 1H, Ar**H**); 6.87–6.97 (m, 2H, Ar**H**); 6.81 (s, 2H, Ar**H**); 6.40 (d, 1H, $J = 15.42$ Hz, olefinic-**H**); 4.86 (d, 1H, $J = 3.67$ Hz, –C**H**–); 4.61–4.76 (m, 1H, –C**H**–); 4.24–4.38 (m, 4H, OC**H2**); 3.92 (s, 6H, OC**H3**); 3.88 (m, 8H, NC**H2**); 3.84 (s, 9H, OC**H3**); 3.16–3.32 (m, 2H, –C**H2**–); 2.63–2.88 (m, 4H, –SC**H2**); 1.76–2.45 (m, 4H, –C**H2**–); 1.56–1.67 (m, 2H, –C**H2**–); 1.22–1.40 (m, 6H, –C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 154.3, 148.7, 143.7, 137.1, 130.1, 129.3, 128.1, 127.3, 126.7, 121.8, 119.7, 112.7, 115.4, 114.0, 112.2, 110.0, 109.4, 108.6, 66.1, 65.1, 60.9, 55.2, 56.3, 52.5, 50.5, 30.9, 29.5, 26.7, 24.7, 22.3, 14.8. ESI-MS: m/z 897 [M$^+$]

*4.1.12. (2S)-N-{4-[4-[2-Methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]butyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (**16b**)*

The compound **16b** was prepared following the method described for the compound **16a**, employing compound **10** (456 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(4-bromobutyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15b** (642 mg, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **16b**. Yield 728 mg (80%): $R_f = 0.61$ (MeOH–$CHCl_3$ 2:98); Light yellow solid. mp: 127–129 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.67 (d, 1H, $J = 14.57$ Hz olefinic-**H**); 7.38 (s, 1H, Ar**H**); 7.01–7.14 (m, 2H, Ar**H**); 6.80–6.93 (m, 3H, Ar**H** and olefinic-**H**); 6.64–6.76 (m, 2H, Ar**H**);

4.88 (d, 1H, J = 3.39 Hz, −C**H**−); 4.68–4.75 (m, 1H, −C**H**−); 4.13–4.26 (m, 4H, OC**H2**); 3.92 (s, 6H, OC**H3**); 3.88 (m, 9H, OC**H3**); 3.69–3.83 (m, 8H, NC**H2**); 3.19–3.33 (m, 2H, −C**H2**−); 2.67–2.88 (m, 4H, −C**H2**−); 2.23–2.34 (m, 2H, −C**H2**−); 2.04–2.15 (m, 2H, −C**H2**−); 1.91–2.02 (m, 2H, −C**H2**−); 1.75–1.86 (m, 2H, −C**H2**−); 1.23–1.38 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.5, 166.1, 165.2, 154.0, 152.7, 149.3, 148.8, 147.7, 142.7, 138.2, 136.2, 130.1, 128.7, 127.8, 121.8, 115.2, 113.2, 111.9, 109.6, 108.7, 107.9, 103.7, 68.2, 67.8, 60.3, 55.6, 52.1, 59.2, 35.5, 29.0, 26.6, 25.5, 24.0, 14.6. ESI-MS: m/z 911 [M^+]

4.1.13. (2S)-N-{4-[5-[2-Methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]pentoxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (**16c**)

The compound **16c** was prepared following the method described for the compound **16a**, employing compound **10** (456 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(5-bromopentoxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15c** (658 mg, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **16c**. Yield 721 mg (78%); R_f = 0.61 (MeOH–$CHCl_3$ 2:98); Light yellow solid. mp: 124–126 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.67 (d, 1H, J = 14.92 Hz, olefinic-**H**); 7.51 (s, 2H, Ar**H**); 7.04 (s, 1H, Ar**H**); 6.88–6.96 (m, 2H, Ar**H**); 6.83 (s, 2H, Ar**H**); 6.42 (d, 1H, J = 15.39 Hz, olefinic-**H**); 4.85 (d, 1H, J = 3.64 Hz, −C**H**−); 4.62–4.77 (m, 1H, −C**H**−); 4.21–4.36 (m, 4H, −C**H2**−); 3.93 (s, 6H, OC**H3**); 3.89 (m, 8H, NC**H2**); 3.85 (s, 9H, OC**H3**); 3.15–3.33 (m, 2H, −C**H2**−); 2.64–2.87 (m, 4H, −C**H2**−); 1.77–2.48 (m, 4H, −C**H2**−); 1.50–1.72(m, 4H, −C**H2**−); 1.21–1.42 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.8, 159.6, 153.6, 147.4, 144.5, 136.9, 131.3, 129.8, 128.5, 127.9, 126.2, 122.3, 120.3, 115.7, 114.6, 112.5, 112.4, 110.2, 109.8, 108.2, 66.7, 65.4, 60.5, 56.8, 53.3, 50.7, 31.7, 29.8, 26.8, 24.9, 22.3, 21.0, 15.4, 14.8. ESI-MS: m/z 925 [M^+]

4.1.14. (2S)-N-{4-[6-[2-Methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]hexyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (**16d**)

The compound **16d** was prepared following the method described for the compound **16a**, employing compound **10** (456 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(6-bromohexyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15d** (675 mg, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **16d**. Yield 694 mg (74%); R_f = 0.65 (MeOH–$CHCl_3$ 2:98); Light yellow solid mp: 121–123 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66 (d, 1H, J = 14.87 Hz, olefinic-**H**); 7.41 (s, 1H, Ar**H**); 7.03–7.14 (m, 2H, Ar**H**); 6.83–6.94 (m, 3H, Ar**H**); 6.62–6.75 (m, 2H, Ar**H** and olefinic-**H**); 4.87 (d, 1H, J = 3.37 Hz, −C**H**−); 4.64–4.73 (m, 1H, −C**H**−); 4.18–4.29 (m, 4H, −C**H2**−); 3.91 (s, 6H, OC**H3**); 3.89 (m, 9H, OC**H3**); 3.68–3.84 (m, 8H, NC**H2**); 3.20–3.34 (m, 2H, −C**H2**−); 2.68–2.89 (m, 4H, −C**H2**−); 2.25–2.37 (m, 2H, −C**H2**−); 2.06–2.17 (m, 4H, −C**H2**−); 1.90–2.05 (m, 4H, −C**H2**−); 1.75–1.86 (m, 2H, −C**H2**−); 1.23–1.38 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2, 167.2, 164.8, 153.9, 152.4, 150.2, 149.8, 148.7, 143.1, 137.8, 136.6, 131.2, 128.3, 127.4, 122.1, 115.6, 113.7, 112.7, 109.3, 108.4, 107.5, 103.6, 68.6, 67.5, 60.1, 55.4, 52.5, 51.2, 36.5, 29.8, 26.3, 25.7, 24.8, 24.2, 23.0, 14.6. ESI-MS: m/z 939 [M^+]

4.1.15. (2S)-N-{4-[3-[2-Methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]propoxy}-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethylthioacetal (**17a**)

To a stirred solution of (*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-morpholino-2-propen-1-one **11** (526 mg, 1 mmol) in dry acetonitrile (20 mL) was added anhydrous K_2CO_3 (1.08 g, 5.0 mmol) and compound (2*S*)-*N*-[4-(3-bromopropoxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethyl thioacetal **15a** (1.25 g, 1.2 mmol). The reaction mixture was stirred at reflux temperature for 12 h and the reaction was monitored by TLC. After completion of the reaction, K_2CO_3 was removed by suction filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (99:1 $CHCl_3$–MeOH) to afford the pure compound **17a**.Yield: 1.09 g, (78%); R_f = 0.63 (MeOH–$CHCl_3$ 1:99); Light yellow solid. mp: 137–139 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.71 (s, 1H, Ar**H**); 7.60 (d, 1H, J = 15.86 Hz, olefinic-**H**); 7.06 (d, 1H, J = 8.30 Hz, Ar**H**); 7.0 (s, 1H, Ar**H**); 6.87 (d, 1H, J = 8.30 Hz, Ar**H**); 6.77 (s, 1H, Ar**H**); 6.66 (d, 1H, J = 15.10 Hz, olefinic-**H**); 4.84 (d, 1H, J = 3.77 Hz, −C**H**−); 4.65–4.72 (m, 1H, −C**H**−); 4.35 (t, 2H, J = 6.04 Hz, OC**H2**−); 4.26 (t, 2H, J = 6.04 Hz, OC**H2**−) 3.94 (s, 3H, OC**H3**); 3.89 (s, 3H, OC**H3**); 3.61–3.77 (m, 8H, NC**H2**); 3.16–3.33 (m, 2H, −C**H2**−); 2.64–2.86 (m, 4H, −C**H2**−); 2.24–2.45 (m, 2H, −C**H2**−); 1.75–2.15 (m, 2H, −C**H2**−); 1.43–1.58 (m, 2H, −C**H2**−); 1.23–1.41 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.7, 165.7, 154.4, 149.9, 149.2, 148.1, 143.0, 137.2, 128.3, 121.8, 114.7, 112.9, 110.3, 109.2, 108.1, 66.8, 65.8, 64.9, 61.1, 56.4, 55.9, 52.9, 50.9, 28.9, 26.8, 24.6, 15.0. ESI-MS: m/z 704 [M^+]

4.1.16. (2S)-N-{4-[4-[2-Methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]butyloxy}-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethylthioacetal (**17b**)

The compound **17b** was prepared following the method described for the compound **17a**, employing compound **11** (526 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(4-bromobutyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15b** (1.28 g, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **17b**. Yield 1.06 g (74%); R_f = 0.63 (MeOH–$CHCl_3$ 1:99); Light yellow solid. mp: 134–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.69 (s, 1H, Ar**H**); 7.64 (d, 1H, J = 15.26 Hz, olefinic-**H**); 7.09 (d, 1H, J = 6.78 Hz, Ar**H**); 7.04 (s, 1H, Ar**H**); 6.87 (d, 1H, J = 8.46 Hz, Ar**H**); 6.81 (s, 1H, Ar**H**); 6.71 (d, 1H, J = 15.26 Hz, olefinic-**H**); 4.87 (d, 1H, J = 3.39 Hz, −C**H**−); 4.68–4.74 (m, 1H, −C**H**−); 4.13–4.26 (m, 4H, OC**H2**−); 3.92 (s, 3H, OC**H3**); 3.89 (s, 3H, OC**H3**); 3.64–3.81 (m, 8H, NC**H2**); 3.18–3.34 (m, 2H, −C**H2**−); 2.67–2.88 (m, 4H, −C**H2**−); 1.92–2.33 (m, 6H, −C**H2**−); 1.73–1.87 (m, 2H, −C**H2**−); 1.29–1.42 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.8, 166.0, 154.2, 149.9, 149.4, 148.4, 142.9, 137.0, 128.3, 121.8, 114.3, 112.2, 110.0, 109.0, 107.7, 69.3, 68.3, 66.7, 60.9, 56.5, 55.7, 52.9, 49.9, 45.8, 27.1, 26.0, 24.5, 14.8. ESI-MS: m/z 718 [M^+]

4.1.17. (2S)-N-{4-[5-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]pentyloxy}-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethylthioacetal (**17c**)

The compound **17c** was prepared following the method described for the compound **17a**, employing compound **11** (526 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(5-bromopentyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15c** (1.31 g, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **17c**. Yield 1.05 g (72%); R_f = 0.61 (MeOH–$CHCl_3$ 1:99); Light yellow solid. mp: 131–133 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.71 (s, 1H, Ar**H**); 7.62 (d, 1H, J = 15.41 Hz, olefinic-**H**); 7.04 (d, 1H, J = 8.10 Hz, Ar**H**); 7.03 (s, 1H, Ar**H**); 6.86 (d, 1H, J = 8.10 Hz, Ar**H**); 6.75 (s, 1H, Ar**H**); 6.64 (d, 1H, J = 15.41 Hz, olefinic-**H**); 4.84 (d, 1H, J = 3.75 Hz, −C**H**−); 4.63–4.71 (m, 1H, −C**H**−); 4.34 (t, 2H, J = 6.32 Hz, OC**H2**−); 4.27 (t, 2H, J = 6.32 Hz, OC**H2**−) 3.93 (s, 3H, OC**H3**); 3.90 (s, 3H, OC**H3**); 3.59–3.75 (m, 8H, NC**H2**−); 3.15–3.35 (m, 2H, −C**H2**−); 2.61–2.84 (m, 4H, −C**H2**−); 2.25–2.47 (m, 2H, −C**H2**−); 1.78–2.17

(m, 4H, −C**H2**−); 1.40–1.56 (m, 4H, −C**H2**−); 1.22–1.43 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.2, 165.3, 154.7, 150.7, 149.7, 148.4, 143.6, 137.5, 128.6, 121.4, 114.6, 112.7, 110.4, 109.5, 108.4, 66.7, 65.5, 64.8, 60.9, 56.6, 55.6, 52.7, 51.2, 28.7, 26.9, 24.8, 24.3, 23.2, 15.0. ESI-MS: *m*/*z* 732 [M^+]

*4.1.18. (2S)-N-{4-[6-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]hexyloxy}-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethylthioacetal **(17d)***

The compound **17d** was prepared following the method described for the compound **17a**, employing compound **11** (526 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(6-bromohexyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15d** (1.35 g, 1.2 mmol) and the crude product was purified by column chromatography to afford the compound **17d**. Yield 1.11 g (75%); R_f = 0.64 (MeOH–$CHCl_3$ 1:99); Light yellow solid. mp: 129–131 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.70 (s, 1H, Ar**H**); 7.60 (d, 1H, *J* = 15.36 Hz, olefinic-**H**); 7.07 (d, 1H, *J* = 6.23 Hz, Ar**H**); 7.05 (s, 1H, Ar**H**); 6.87 (d, 1H, *J* = 8.26 Hz, Ar**H**); 6.80 (s, 1H, Ar**H**); 6.74 (d, 1H, *J* = 15.36 Hz, olefinic-**H**); 4.85 (d, 1H, *J* = 3.34 Hz, −C**H**−); 4.66–4.72 (m, 1H, −C**H**−); 4.15–4.27 (m, 4H, OC**H2**−); 3.92 (s, 3H, OC**H3**); 3.88 (s, 3H, OC**H3**); 3.60–3.82 (m, 8H, NC**H2**); 3.19–3.36 (m, 2H, −C**H2**−); 2.67–2.89 (m, 4H, −C**H2**−); 1.93–2.31 (m, 6H, −C**H2**−); 1.52–1.88 (m, 6H, −C**H2**−); 1.25–1.44 (m, 6H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.6, 165.7, 154.6, 150.7, 149.1, 148.7, 143.0, 137.3, 128.7, 121.4, 114.7, 112.6, 110.3, 109.2, 107.5, 69.6, 68.2, 66.5, 60.7, 56.8, 55.4, 52.5, 49.6, 45.6, 28.3, 26.1, 24.5, 24.3, 23.6, 14.8. ESI-MS: *m*/*z* 746 [M^+]

*4.1.19. (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-propoxy-3-methoxy phenyl)prop-2-en-1-one dioxy]-bis [(2-nitro-5-methoxy-1,4-phenylene)carbonyl]-bis[pyrrolidine-2-carboxaldehyde diethylthioacetal] **(20a)***

To a stirred solution of compound **12** (439 mg, 1 mmol) in dry acetonitrile (20 mL) was added anhydrous K_2CO_3 (1.3 g, 5.0 mmol) and compound (2*S*)-*N*-[4-(3-bromopropoxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15a** (1.08 g, 2 mmol). The reaction mixture was stirred at reflux temperature for 12 h and the reaction was monitored by TLC. After completion of the reaction, K_2CO_3 was removed by suction filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (96:4 $CHCl_3$–MeOH) to afford the pure compound **20a.** Yield: 1.0 g, (76%); R_f = 0.64 (MeOH–$CHCl_3$ 4:96); Light yellow solid. mp: 140–142 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.72 (s, 2H, Ar**H**), 7.65 (d, 2H, *J* = 15.10 Hz, olefinic-**H**), 6.99–7.15 (m, 4H, Ar**H**), 6.78–6.94 (m, 4H, Ar**H**), 6.72 (d, 2H, *J* = 15.86 Hz, olefinic-**H**), 4.87 (d, 2H, *J* = 3.77 Hz, −C**H**−), 4.63–4.76 (m, 2H, −C**H**−), 4.16–4.48 (m, 8H, OC**H2**−), 3.91 (s, 12H, OC**H3**), 3.58–3.84 (m, 8H, NC**H2**−), 3.12–3.33 (m, 4H, −C**H2**−), 2.63–2.89 (m, 8H, −C**H2**−), 1.70–2.45 (m, 2H, −C**H2**−), 115–1.48 (m, 12H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.1, 149.6, 149.3, 148.0, 143.4, 137.0, 129.2, 128.0, 121.7, 114.0, 112.5, 110.0, 109.0, 108.2, 65.8, 64.9, 60.9, 56.3, 52.7, 50.1, 31.5, 28.7, 26.5, 24.5, 22.6, 14.9. ESI-MS: *m*/*z* 1319 [M^+]

*4.1.20. (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-butyloxy-3-methoxy phenyl-)prop-2-en-1-one dioxy]-bis [(2-nitro-5-methoxy-1,4-phenylene)carbonyl]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] **(20b)***

The compound **20b** was prepared following the method described for the compound **20a**, employing compound **12** (439 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(4-bromobutyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15b** (1.11 g, 2 mmol) and the crude product was purified by column chromatography to afford the compound **20b**. Yield 984 mg (73%); R_f = 0.64 (MeOH–$CHCl_3$ 4:96); Light yellow solid. mp: 138–140 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.71 (s, 2H, Ar**H**), 7.64 (d, 2H, *J* = 15.30 Hz, olefinic-**H**), 6.97–7.14 (m, 4H, Ar**H**), 6.79–6.96 (m, 4H, Ar**H**), 6.73 (d, 2H, *J* = 15.46 Hz, olefinic-**H**), 4.84 (d, 2H, *J* = 3.77 Hz, −C**H**−), 4.61–4.74 (m, 2H, −C**H**−), 4.17–4.47 (m, 8H, OC**H2**−), 3.90 (s, 12H, OC**H3**), 3.56–3.84 (m, 8H, NC**H2**−), 3.11–3.32 (m, 4H, −C**H2**−), 2.62–2.87 (m, 8H, −C**H2**−), 1.72–2.44 (m, 6H, −C**H2**−), 116–1.47 (m, 12H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.3, 150.1, 149.6, 147.7, 143.6, 136.8, 129.5, 128.4, 121.4, 114.6, 112.8, 110.3, 109.7, 108.5, 66.7, 64.6, 60.8, 56.5, 52.7, 50.3, 31.8, 28.4, 26.9, 24.8, 23.4, 22.8, 15.2. ESI-MS: *m*/*z* 1347 [M^+].

*4.1.21. (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-pentyloxy-3-methoxy-phenyl-)prop-2-en-1-one dioxy]-bis [(2-nitro-5-methoxy-1,4-phenylene)carbonyl]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] **(20c)***

The compound **20c** was prepared following the method described for the compound **20a**, employing compound **12** (439 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(5-bromopentyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15c** (1.13 g, 2 mmol) and the crude product was purified by column chromatography to afford the compound **20c**. Yield 1.01 g (74%); R_f = 0.62 (MeOH–$CHCl_3$ 4:96); Light yellow solid. mp: 134–136 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.69 (s, 2H, Ar**H**), 7.63 (d, 2H, *J* = 15.17 Hz, olefinic-**H**), 6.98–7.16 (m, 4H, Ar**H**), 6.76–6.92 (m, 4H, Ar**H**), 6.74 (d, 2H, *J* = 15.47 Hz, olefinic-**H**), 4.85 (d, 2H, *J* = 3.77 Hz, −C**H**−), 4.62–4.75 (m, 2H, −C**H**−), 4.18–4.49 (m, 8H, OC**H2**−), 3.92 (s, 12H, OC**H3**), 3.57–3.86 (m, 8H, NC**H2**), 3.14–3.35 (m, 4H, −C**H2**−), 2.64–2.88 (m, 8H, −C**H2**−), 1.67–2.48 (m, 6H, −C**H2**−), 114–1.47 (m, 12H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.1, 149.8, 148.9, 147.6, 143.2, 137.5, 129.6, 128.2, 121.5, 114.3, 112.4, 110.3, 109.7, 108.6, 65.9, 64.7, 61.2, 56.7, 52.4, 50.3, 31.6, 28.8, 27.4, 26.3, 24.9, 24.1, 23.8, 15.1. ESI-MS: *m*/*z* 1375 [M^+]

*4.1.22. (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-hexyloxy-3-methoxy- phenyl-)prop-2-en-1-one dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] **(20d)***

The compound **20d** was prepared following the method described for the compound **20a**, employing compound **12** (439 mg, 1 mmol) and the compound (2*S*)-*N*-[4-(6-bromohexyloxy-5-methoxy-2-nitrobenzoyl)]pyrrolidine-2-carboxaldehyde diethylthioacetal **15d** (1.16 g, 2 mmol) and the crude product was purified by column chromatography to afford the compound **20d**. Yield 1.05 g (75%); R_f = 0.60 (MeOH–$CHCl_3$ 4:96); Light yellow solid. mp: 132–134 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.72 (s, 2H, Ar**H**), 7.66 (d, 2H, *J* = 15.48 Hz, olefinic-**H**), 6.97–7.13 (m, 4H, Ar**H**), 6.78–6.95 (m, 4H, Ar**H**), 6.72 (d, 2H, *J* = 15.44 Hz, olefinic-**H**), 4.86 (d, 2H, *J* = 3.77 Hz, −C**H**−), 4.64–4.77 (m, 2H, −C**H**−), 4.20–4.49 (m, 8H, OC**H2**−), 3.91 (s, 12H, −OC**H3**), 3.57–3.85 (m, 8H, NC**H2**−), 3.13–3.35 (m, 4H, −C**H2**−), 2.61–2.87 (m, 8H, −C**H2**−), 1.63–2.45 (m, 8H, −C**H2**−), 117–1.46 (m, 12H, −C**H3**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.0, 149.4, 148.7, 148.1, 143.5, 137.3, 129.4, 128.6, 121.5, 114.4, 112.3, 110.5, 109.3, 108.7, 66.8, 64.7, 61.0, 56.4, 52.6, 50.5, 31.6, 28.9, 27.3, 26.5, 24.9, 23.8, 23.0, 21.4, 14.9. ESI-MS: *m*/*z* 1403 [M^+]

*4.1.23. General procedure for the synthesis of compounds **18a–d**, **19a–d** and **21a–d***

The Nitro compound (**16a–d, 17a–d** and **20a–d**) (1 mmol) dissolved in methanol (30 mL) and added $SnCl_2 \cdot 2H_2O$ (4 mmol) was refluxed for 2 h or until the TLC indicated that reaction was complete. The methanol was evaporated under vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with EtOAc (2 × 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under

vacuum to afford the corresponding amino compounds (**18a**–**d**, **19a**–**d** and **21a**–**d**) as yellow liquid, which due to potential stability problems, directly used in the next step.

*4.1.24. 7-Methoxy-8-{3-[2-methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]propoxy}-(11 aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(4a)***

A solution of **18a** (867 mg, 1 mmol), $HgCl_2$ (693 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in $CH_3CN{\cdot}H_2O$ (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a celitebed. The clear yellow organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried on Na_2SO_4. The organic layer was evaporated under vacuum and purified by column chromatography (95% $CHCl_3{\cdot}CH_3OH$) to give compound **4a**. This material was repeatedly evaporated from $CHCl_3$ under vacuum to generate the imine form. Yield 482 mg (65%); R_f = 0.56 (MeOH- $CHCl_3$ 5:95); White solid. mp: 118–120 °C; $[\alpha]_D^{25}$ = +255 (c = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.61–7.69 (m, 2H, imine-**H** and olefinic-**H**); 7.50 (s, 1H, Ar**H**); 6.98–7.13 (m, 2H, Ar**H**); 6.82–6.93 (m, 3H, Ar**H**); 6.63–6.76 (m, 2H, Ar**H** and olefinic-**H**); 4.20–4.36 (m, 4H, OC**H2**–); 3.91 (s, 6H, OC**H3**); 3.88 (s, 9H, OC**H3**); 3.65–3.85 (m, 9H, NC**H2**, –C**H**–); 3.50–3.63 (m, 2H, –C**H2**–); 2.26–2.45 (m, 2H, –C**H2**–); 1.98–2.12 (m, 2H, –C**H2**–); 1.63–1.80 (m, 2H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 165.8, 164.5, 162.3, 153.3, 150.6, 150.0, 149.5, 147.6, 143.6, 140.3, 139.5, 130.3, 127.8, 121.7, 120.2, 113.9, 112.6, 111.5, 110.6, 110.4, 65.2, 60.7, 56.3, 55.8, 53.6, 46.6, 31.4, 29.4, 28.7, 23.8.; ESI-MS: m/z 743 [M^+]; HRMS (ESI): [M + Na]$^+$ calcd for $C_{40}H_{47}N_4O_{10}$ m/z 743.3327, found m/z 743.3292; IR (KBr) (U_{max}/cm^{-1}): 3412, 2932, 1592, 1510, 1460, 1425, 1329, 1257, 1127, 1023, 812, 763..

*4.1.25. 7-Methoxy-8-{4-[2-methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]butyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(4b)***

The compound **4b** was prepared following the method described for the compound **4a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **4b**. Yield 469 mg (62%); R_f = 0.56 (MeOH–$CHCl_3$ 5:95); White solid. mp: 113–115 °C; $[\alpha]_D^{25}$ = +235 (c = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.62–7.71 (m, 2H, imine-**H** and olefinic-**H**); 7.49 (s, 1H, Ar**H**); 6.99–7.15 (m, 2H, Ar**H**); 6.80–6.92 (m, 3H, Ar**H**); 6.61–6.67 (m, 2H, Ar**H** and olefinic-**H**); 4.07–4.24 (m, 4H, OC**H2**–); 3.91 (s, 6H, OC**H3**); 3.86 (s, 9H, OC**H3**); 3.46–3.82 (m, 11H, NC**H2**, –C**H2**– and –C**H**–); 2.24–2.39 (m, 2H, –C**H2**–); 1.92–2.14 (m, 4H, –C**H2**–); 1.17–1.34 (m, 2H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 168.8, 166.1, 164.6, 162.3, 153.2, 150.3, 149.2, 143.2, 140.1, 139.2, 130.9, 129.4, 127.8, 121.8, 119.6, 113.8, 112.5, 111.3, 110.2, 104.2, 68.1, 60.6, 56.1, 53.2, 46.3, 31.6, 29.0, 25.4, 24.0, 22.3, 14.0.; ESI-MS: m/z 757 [M^+]; IR (KBr) (U_{max}/cm^{-1}): 3383, 2927, 1587, 1510, 1462, 1425, 1329, 1258, 1169, 1126, 1019, 843, 763.

*4.1.26. 7-Methoxy-8-{5-[2-methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]pentyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(4c)***

The compound **4c** was prepared following the method described for the compound **4a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **4c**. Yield 462 mg (60%); R_f = 0.56 (MeOH–$CHCl_3$ 5:95); White solid. mp: 112–114 °C; $[\alpha]_D^{25}$ = +274 (c = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63–7.75 (m, 2H, imine-**H** and olefinic-**H**); 7.50 (s, 1H, Ar**H**); 6.98–7.16 (m, 2H, Ar**H**); 6.82–6.93 (m, 3H, Ar**H**); 6.62–6.68 (m, 2H, Ar**H** and olefinic-**H**); 4.10–4.25 (m, 4H, OC**H2**–); 3.91 (s, 6H, OC**H3**); 3.87 (s, 9H, OC**H3**); 3.45–3.86 (m, 11H, NC**H2**, –C**H2**– and –C**H**–); 2.25–2.45 (m, 2H, –C**H2**–); 1.91–2.17 (m, 4H, –C**H2**–); 1.16–1.38 (m, 4H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 166.2, 164.3, 162.7, 154.6, 151.2, 150.5, 149.4, 148.1, 143.4, 140.7, 139.4, 130.7, 127.6, 121.5, 120.4, 114.0, 112.8, 111.7, 110.5, 110.8, 66.7, 60.8, 56.7, 55.3, 53.7, 46.3, 31.8, 29.9, 28.7, 25.4, 24.5, 23.8.; ESI-MS: m/z 771 [M^+]; IR (KBr) (U_{max}/cm^{-1}): 3419, 2941, 1578, 1510, 1465, 1426, 1330, 1256, 1150, 1025, 824, 764.

*4.1.27. 7-Methoxy-8-{6-[2-methoxy-4-(E)-3-oxo-3-[4-(3,4,5-trimethoxybenzoyl)piperazino]-1-propenylphenoxy]hexyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(4d)***

The compound **4d** was prepared following the method described for the compound **4a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **4d**. Yield 478 mg (61%); R_f = 0.53 (MeOH–$CHCl_3$ 5:95); White solid.mp: 109–111 °C; $[\alpha]_D^{25}$ = +247 (c = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.61–7.72 (m, 2H, imine-**H** and olefinic-**H**); 7.52 (s, 1H, Ar**H**); 6.99–7.18 (m, 2H, Ar**H**); 6.81–6.95 (m, 3H, Ar**H**); 6.63–6.69 (m, 2H, Ar**H** and olefinic-**H**); 4.12–4.25 (m, 4H, OC**H2**–); 3.92 (s, 6H, OC**H3**); 3.88 (s, 9H, OC**H3**); 3.46–3.87 (m, 11H, NC**H2**, –C**H2**– and –C**H**–); 2.24–2.47 (m, 2H, –C**H2**–); 1.92–2.18 (m, 4H, –C**H2**–); 1.14–1.46 (m, 6H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 167.5, 166.5, 164.3, 162.7, 153.2, 151.8, 149.4, 143.6, 140.4, 139.3, 131.5, 129.7, 127.6, 121.5, 119.2, 113.1, 112.8, 111.6, 110.7, 104.6, 68.7, 60.4, 56.5, 53.7, 46.5, 31.5, 29.7, 25.8, 24.7, 24.1, 23.0, 22.3, 14.0.; ESI-MS: m/z 785 [M^+]; IR (KBr) (U_{max}/cm^{-1}): 3397, 2929, 1584, 1512, 1464, 1425, 1329, 1258, 1169, 1122, 1020, 828, 763.

*4.1.28. 7-Methoxy-8-{3-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]propoxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(5a)***

A solution of **19a** (741 mg, 1 mmol), $HgCl_2$ (740 mg, 2.26 mmol) and $CaCO_3$ (270 mg, 2.46 mmol) in $CH_3CN{\cdot}H_2O$ (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a celitebed. The clear yellow organic supernatant was extracted with saturated 5% NaHCO3 (20 mL), brine (20 mL) and the combined organic phase was dried on Na2SO4. The organic layer was evaporated in vacuum and purified by column chromatography (95% $CHCl_3$–CH3OH) to give compound **5a.** This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. Yield: 368 mg, 61%; R_f = 0.58 (MeOH–$CHCl_3$ 4:95); White solid. mp: 110–112 °C; $[\alpha]_D^{25}$ = +355 (c = 0.5 in $CHCl_3$);^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.60–7.67 (m, 2H, imine-**H** and olefinic-**H**); 7.51 (s, 1H, Ar**H**); 7.08 (d, 1H, J = 7.77 Hz, Ar**H**); 7.01 (s, 1H, Ar**H**); 6.90 (d, 1H, J = 8.63 Hz, Ar**H**); 6.85 (s, 1H, Ar**H**); 6.69 (d, 1H, J = 14.67 Hz, olefinic-**H**); 4.20–4.36 (m, 4H, OC**H2**–); 3.92 (s, 3H, OC**H3**); 3.88 (s, 3H, OC**H3**); 3.51–3.86 (m, 11H, morpholine–C**H2**, –C**H2**– and –C**H**–); 2.24–2.45 (m, 2H, –C**H2**–); 1.98–2.13 (m, 2H, –C**H2**–); 1.69–1.84 (m, 2H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.7, 164.5, 162.3, 150.5, 149.9, 149.5, 147.7, 143.1, 140.5, 128.2, 121.6, 120.2, 114.2, 112.9, 111.6, 110.7, 66.8, 65.3, 56.0, 53.6, 46.5, 45.9, 42.3, 29.5, 28.9, 24.1.; ESI-MS: m/z 550 [M^+]; HRMS (ESI): [M + Na]$^+$ calcd for C30H36 N3O7 m/z 550.2566, found m/z 550.2553; IR (KBr) (U_{max}/cm^{-1}): 3420, 2958, 1641, 1599, 1510, 1459, 1432, 1336, 1262, 1166, 1139, 1116, 980, 870, 762.

*4.1.29. 7-Methoxy-8-{4-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]butyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(5b)***

The compound **5b** was prepared following the method described for the compound **5a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **5b**. Yield: 396 mg, (64%); R_f = 0.58 (MeOH–$CHCl_3$ 4:95); White solid. mp: 108–110 °C; $[\alpha]_D^{25}$ = +372 (c = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.62–7.68 (m, 2H, imine-**H** and olefinic-**H**); 7.51 (s, 1H, Ar**H**); 7.09 (d, 1H, J = 7.77 Hz, Ar**H**); 7.02 (s, 1H, Ar**H**); 6.87 (d, 1H, J = 8.63 Hz, Ar**H**); 6.82 (s, 1H, Ar**H**); 6.70 (d, 1H, J = 14.67 Hz, olefinic-**H**); 4.07–4.25 (m, 4H, OC**H2**–); 3.92 (s, 3H, OC**H3**); 3.88 (s, 3H, OC**H3**); 3.53–3.86 (m, 11H, morpholine–C**H2**, –C**H2**– and –C**H**–); 2.27–2.38 (m, 2H, –C**H2**–); 1.98–2.17 (m, 4H, –C**H2**–); 1.67–1.82 (m, 2H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.7, 164.5, 162.2, 150.6, 150.0, 149.3, 147.6, 143.2, 140.4, 127.9, 121.7, 120.0, 114.0, 112.3, 111.3, 110.2, 68.4, 55.9, 53.6, 46.5, 42.5, 29.5, 25.7, 25.6, 24.0.; ESI-MS: m/z 564 [M^+]; HRMS (ESI): $[M + Na]^+$ calcd for C31H38 N3O7 m/z 564.2719, found m/z 564.2709; IR (KBr) (U_{max}/cm^{-1}): 3366, 2954, 1640, 1598, 1511, 1433, 1337, 1262, 1138, 1116, 1037, 979, 869, 806.

*4.1.30. 7-Methoxy-8-{5-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]pentyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(5c)***

The compound **5c** was prepared following the method described for the compound **5a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **5c**. Yield: 380 mg, 60%; R_f = 0.58 (MeOH–$CHCl_3$ 4:95); White solid. mp: 107–109 °C; $[\alpha]_D^{25}$ = +337 (c = 0.5 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.65–7.70 (m, 2H, imine-**H** and olefinic-**H**); 7.50 (s, 1H, Ar**H**); 7.06 (d, 1H, J = 7.75 Hz, Ar**H**); 7.0 (s, 1H, Ar**H**); 6.91 (d, 1H, J = 8.66 Hz, Ar**H**); 6.84 (s, 1H, Ar**H**); 6.70 (d, 1H, J = 14.81 Hz, olefinic-**H**); 4.21–4.38 (m, 4H, OC**H2**–); 3.92 (s, 3H, OC**H3**); 3.89 (s, 3H, OC**H3**); 3.50–3.85 (m, 11H, morpholine–C**H2**, –C**H2**– and –C**H**–); 2.23–2.46 (m, 2H, –C**H2**–); 1.87–2.16 (m, 4H, –C**H2**–); 1.66–1.83 (m, 4H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.4, 164.6, 162.6, 150.4, 149.6, 149.1, 147.5, 143.3, 140.7, 128.6, 121.4, 120.5, 114.6, 112.7, 111.3, 110.5, 66.7, 65.4, 56.3, 53.7, 46.8, 46.1, 42.8, 29.8, 28.5, 26.4, 24.8, 24.1.; ESI-MS: m/z 578 [M^+]; IR (KBr) (U_{max}/cm^{-1}): 3412, 2956, 1642, 1597, 1512, 1446, 1434, 1325, 1264, 1146, 1135, 1115, 978, 871, 764.

*4.1.31. 7-Methoxy-8-{6-[2-methoxy-4-[(E)-3-morpholino-3-oxo-1-propenyl]phenoxy]hexyloxy}-(11aS)1,2,3,11a-tetra-hydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one **(5d)***

The compound **5d** was prepared following the method described for the compound **5a**, employing $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) and the crude product was purified by column chromatography to afford the compound **5d**. Yield: 389 mg, (60%); R_f = 0.56 (MeOH–$CHCl_3$ 4:95); White solid. mp: 102–104 °C; $[\alpha]_D^{25}$ = +355 (c = 0.5 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.64–7.69 (m, 2H, imine-**H** and olefinic-**H**); 7.49 (s, 1H, Ar**H**); 7.05 (d, 1H, J = 7.15 Hz, Ar**H**); 7.01 (s, 1H, Ar**H**); 6.86 (d, 1H, J = 8.33 Hz, Ar**H**); 6.83 (s, 1H, Ar**H**); 6.69 (d, 1H, J = 14.81 Hz, olefinic-**H**); 4.15–4.26 (m, 4H, OC**H2**–); 3.91 (s, 3H, OC**H3**); 3.89 (s, 3H, OC**H3**); 3.52–3.87 (m, 11H, morpholine–C**H2**, –C**H2**– and –C**H**–); 2.26–2.37 (m, 2H, –C**H2**–); 1.93–2.13 (m, 6H, –C**H2**–); 1.64–1.81 (m, 4H, –C**H2**–). ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.1, 164.7, 162.4, 151.1, 150.3, 149.5, 147.7, 143.4, 140.6, 127.6, 121.8, 120.4, 114.2, 112.5, 111.7, 110.4, 68.7, 56.2, 53.8, 46.6, 42.3, 29.7, 26.5, 25.8, 24.7, 24.2, 23.4.; ESI-MS: m/z 592 [M^+]; IR (KBr) (U_{max}/cm^{-1}): 3386, 2956, 1643, 1597, 1510, 1439, 1339, 1264, 1137, 1116, 1036, 978, 866, 804.

*4.1.32 (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-propoxy-3-methoxy-phenyl)prop-2-en-1-one dioxy]- bis[(11aS)-7-methoxy-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one] **(6a)***

A solution of **21a** (644 mg, 1 mmol), $HgCl_2$ (1.8 g, 4.52 mmol) and $CaCO_3$ (540 mg, 4.98 mmol) in $CH_3CN \cdot H_2O$ (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with EtOAc (25 mL) and filtered through a celitebed. The clear yellow organic supernatant was extracted with saturated 5% NaHCO3 (20 mL), brine (20 mL) and the combined organic phase was dried on NaSO4. The organic layer was evaporated in vacuum and purified by column chromatography (93% $CHCl_3$–CH3OH) to give compound **6a.** This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. Yield: 303 mg, (60%); R_f = 0.56 (MeOH–$CHCl_3$ 7:93); White solid. mp: 125–127 °C; $[\alpha]_D^{25}$ = +125 (c = 0.5 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.59–7.81 (m, 4H, imine-**H** and olefinic-**H**), 7.51 (s, 2H, Ar**H**), 6.67–7.22 (m, 8H, Ar**H**), 6.52 (d, 2H, J = 16.05 Hz, olefinic-**H**), 4.18–4.40 (m, 8H, OC**H2**–), 3.91 (s, 12H, OC**H3**), 3.46–3.87 (m, 11H, NC**H2**, –C**H2**– and –C**H**–), 1.94–2.48 (m, 8H, –C**H2**–), 1.57–1.83 (m, 4H, –C**H2**–). ESI-MS: m/z 1011 [M^+].

*4.1.33 (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-butyloxy-3-methoxy-phenyl-)prop-2-en-1-one dioxy]-bis[(11aS)-7-methoxy-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4] benzodiazepin-5-one] **(6b)***

The compound **6b** was prepared following the method described for the compound **6a**, employing $HgCl_2$ (4.52 mmol) and $CaCO_3$ (4.98 mmol) and the crude product was purified by column chromatography to afford the compound **6b**. Yield: 316 mg, 61%; R_f = 0.58 (MeOH–$CHCl_3$ 7:93); White solid. mp: 121–123 °C; $[\alpha]_D^{25}$ = +122 (c = 0.5 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.56–7.82 (m, 4H, imine-**H** and olefinic-**H**), 7.50 (s, 2H, Ar**H**), 6.65–7.23 (m, 8H, Ar**H**), 6.53 (d, 2H, J = 15.96 Hz, olefinic-**H**), 4.20–4.43 (m, 8H, OC**H2**–), 3.91 (s, 12H, OC**H3**), 3.43–3.88 (m, 11H, NC**H2**, –C**H2**– and –C**H**–), 1.96–2.49 (m, 8H, –C**H2**–), 1.55–1.84 (m, 6H, –C**H2**–). ESI-MS: m/z 1039 [M^+].

*4.1.34 (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-penyloxy-3-methoxy phenyl-)prop-2-en-1-one dioxy]-bis[(11aS)-7-methoxy-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4] benzodiazepin-5-one] **(6c)***

The compound **6c** was prepared following the method described for the compound **6a**, employing $HgCl_2$ (4.52 mmol) and $CaCO_3$ (4.98 mmol) and the crude product was purified by column chromatography to afford the compound **6c**. Yield: 330 mg, (62%); R_f = 0.60 (MeOH–$CHCl_3$ 7:93); White solid. mp: 120–122 °C; $[\alpha]_D^{25}$ = +117 (c = 0.5 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.57–7.80 (m, 4H, imine-**H** and olefinic-**H**), 7.52 (s, 2H, Ar**H**), 6.67–7.24 (m, 8H, Ar**H**), 6.54 (d, 2H, J = 15.87 Hz, olefinic-**H**), 4.19–4.41 (m, 8H, OC**H2**–), 3.90 (s, 12H, OC**H3**), 3.45–3.86 (m, 11H, NC**H2**, –C**H2**– and –C**H**–), 1.95–2.46 (m, 8H, –C**H2**–), 1.56–1.82 (m, 4H, –C**H2**–), 1.35–1.49 (m, 4H, –C**H2**–). ESI-MS: m/z 1067 [M^+].

*4.1.35 (2E,2′E)-1,1′-(Piperazine-1,4-diyl)bis(4-hexyloxy-3-methoxy phenyl-)prop-2-en-1-one dioxy]bis[(11aS)-7-methoxy-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4] benzodiazepin–5-one] **(6d)***

The compound **6d** was prepared following the method described for the compound **6a**, employing $HgCl_2$ (4.52 mmol) and $CaCO_3$ (4.98 mmol) and the crude product was purified by column chromatography to afford the compound **6d**. Yield: 328 mg, 60%; R_f = 0.60 (MeOH–$CHCl_3$ 7:93); White solid. mp: 118–121 °C; [α] D25 = +103 (c = 0.5 in $CHCl_3$); 1H NMR (200 MHz, $CDCl_3$) δ: 7.54–7.79 (m, 4H, imine-**H** and olefinic-**H**), 7.51 (s, 2H, Ar**H**), 6.66–7.21 (m, 8H, Ar**H**), 6.51 (d, 2H, J = 16.04 Hz, olefinic-**H**), 4.17–4.40 (m, 8H, OC**H2**-), 3.91 (s, 12H, OC**H3**), 3.47–3.86 (m, 11H, NC**H2**, –C**H2**– and –C**H**–), 1.93–2.47 (m, 8H, –C**H2**–), 1.57–1.83 (m, 4H, –C**H2**–), 1.28–1.50 (m, 6H, –C**H2**–). ESI-MS: m/z 1095 [M^+].

4.2. Evaluation of anticancer activity

The synthesized compounds (**4a**–**d**, **5a**–**d** and **6a**–**d**) have been evaluated for their in vitro cytotoxicity in human cancer cell lines. A protocol of 48 h continuous drug exposure has been used and a sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine and were inoculated into 96 well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations. For each compound four concentrations (0.1, 1, 10 and 100 uM) were evaluated and each was done in triplicate wells. Plates were incubated further for 48 h and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The plates were washed five times with tap water and airdried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were airdried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate–by plate basis for test wells relative to control wells. The above determinations were repeated three times. Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) × 100. Growth inhibition of 50% (GI50) was calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. Where, Tz = Optical density at time zero, OD of control = C, and OD of test growth in the presence of drug = Ti.

4.3. Isolation of tubulin

Tubulin was purified according to the earlier protocol [54]. In brief fresh bovine brain was obtained from slaughter house under cold conditions and the cerebral hemispheres were dissected immediately, washed in PBS (phosphate buffer saline) and homogenized with appropriate amount of PEM buffer contain 80 mM PIPES, 0.5 mM $MgCl_2$, and 1 mM EGTA. The homogenate was centrifuged for 30 min at 4 °C at 14,500 rpm. The supernatant was collected and centrifuged in an ultra centrifuge at 4 °C for 60 min at 1, 00,000*g*. The obtained pellet was discarded and the supernatant was incubated with 1% glycerol and 3 mg GTP at 37 °C for 30 min to assist the polymerize the tubulin into microtubules. The supernatant was centrifuged in an ultra centrifuge at 25 °C for 60 min at 1,00,000*g*. The pellet (polymerized tubulin) was suspended in ice-cold PEM buffer and incubated on ice for 30 min to depolymerize the tubulin. The suspension (depolymerized tubulin) is again centrifuged in ultra centrifuge at 4 °C for 60 min at 1,00,000*g* and supernatant was collected. This polymerization and depolymerization cycles are repeated for three times to get purified tubulin. The purified tubulin was stored in MT buffer at −80 °C.

4.4. Tubulin polymerization assay

The assay was carried out in a 384 well plate in PEM buffer [100 mM PIPES (pH-6.9), 1 mM MgCl2, 1 mM EGTA, 10% glycerol]. The reaction mixture in a total volume of 10 μL contained PEM buffer, GTP (1 mM) and in the presence or absence of test compounds **5b**, **6b** (2.5 μM). The reaction was initiated by the addition of GTP to all the wells. Polymerization reaction was monitored by the increase in absorbance at 340 nm using Dynex multimode plate reader at 37 °C. Absorbance was recorded at every 2 min intervals for up to 3 h. Nocodazole was used as positive control in each assay. The IC50 value was defined as the drug concentration required to inhibit 50% of tubulin assembly compared to controls. The reaction mixture for these experiments include: tubulin (3 mg/ml) in PEM buffer, GTP (1 mM), in the presence or absence of test compounds **5b**, **6b** at 2.5 μM, 5 μM, 10 μM, and 15 μM concentrations, different concentrations of nocodozole was used as positive control in this study. Polymerization was monitored by increase in the absorbance at 340 nm using Dynex multimode plate reader at37 °C. Absorbance was monitored at every 2 min for 3 h.

4.5. Immunohistochemistry and microscopy

MCF7 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds **5b**, **6b** (2.5 μM). Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS and permiabilized by dipping the cells in 100% methanol (−20 °C). Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 h followed by incubation with a primary anti tubulin (mouse monoclonal) antibody and FITC conjugated secondary mouse anti Ig G antibody. Photographs were taken using the confocal microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, nocodozole (2.5 μM) and taxol (2.5 μM) were used as negative and positive controls for analyzing microtubule integrity.

4.6. Analysis of cell cycle

Breast cancer cells (MCF7) in 6 well plates were incubated for 48 h in the presence or absence of test compounds **5b**, **6b** (2.5 μM), nocodazole & podophyllotoxin of (1 μM). Cells were harvested with Trypsin–EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, ethanol was removed by centrifugation and cells were 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 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.

4.7. Analysis of nuclear morphology

MCF7 cells were seeded on glass cover slip, incubated for 48 h in the presence or absence of test compounds **5b**, **6b** (2.5 μM). Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS; the cells were incubated with the nuclear stain Hoechst, to analyze morphology of nucleus. Photographs were taken in an olyumpus fluorescence microscopy equipped with DAPI filter settings.

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