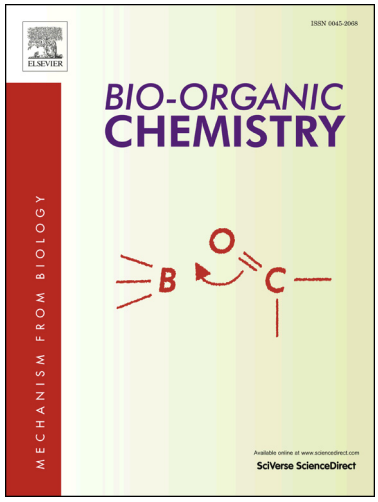

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Dithiocarbamate/Piperazine Bridged Pyrrolobenzodiazepines as DNA-minor Groove Binders: Synthesis, DNA-Binding Affinity and Cytotoxic Activity

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ABSTRACT

A new series of C8-linked dithiocarbamate/piperazine bridged pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates (**5a–c**, **6a,b**) have been synthesized and evaluated for their cytotoxic potential and DNA-binding ability. The representative conjugates **5a** and **5b** have been screened for their cytotoxicity against a panel of 60 human cancer cell lines. Compound **5a** has shown promising cytotoxic activity on selected cancer cell lines that display melanoma, leukemia, CNS, ovarian, breast and renal cancer phenotypes. The consequence of further replacement of the 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate in **5b** and **5c** with 4-methylpiperazine-1-carbodithioate yielded new conjugates **6a** and **6b** respectively. In addition, the compounds **5c** and **6a**,**b** have been evaluated for their *in vitro* cytotoxicity on some of the selected human cancer cell lines and these conjugates have exhibited significant cytotoxic activity. Further, the DNA-binding ability of these new conjugates has been evaluated by using thermal denaturation (ΔT_m) studies. The correlation between structure and DNA-binding ability has been investigated by molecular modeling studies which predicted that **6b** exhibits superior DNA-binding ability and these are in agreement with the experimental DNA-binding studies.

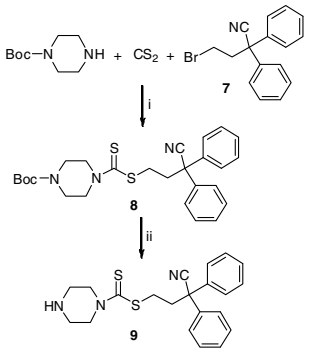
1. Introduction

The minor groove binders are one of the most interesting and widely studied classes of DNA ligands characterized by their sequence specificity. DNA sequence specificity or selectivity has become recognized as an important characteristic of many cytotoxic agents[1,2] such as duocarmycins,[3] distamycin, netropsin[4] and pyrrolo[1,4]benzodiazepines.[5] Several of such molecules are currently in clinical usage for the treatment of human malignancies. Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs), represent a group of exceptionally potent naturally occurring antitumor antibiotics, derived from *Streptomyces* species; examples of which include, DC-81 (**1**, **Fig. 1**), anthramycin, tomaymycin and sibiromycin.[6,7] Their interactions with DNA are unique since they bind within the minor groove of DNA forming a covalent aminal bond between the C11-position of the central B-ring and N2-amino group of a guanine base.[6,7] There is either an imine or carbinolamine methyl ether moiety at the N10–C11 position.[8–11] The latter is an electrophilic center responsible for alkylating DNA. In this context, we have been engaged during the last few years in the structural modifications and development of new synthetic strategies for the PBD based ring system.[12–21]

On the other hand, piperazine motifs are amongst the key scaffolds in today's drug discovery owing to their potential biological properties including anticancer activities.[22] Moreover, the piperazine scaffold occurs frequently in complex natural products.[23,24] Previously, we have designed and synthesized C8-linked *N*-methyl piperazine PBD monomers[25] and their dimers[12] that displayed significant DNA-binding ability and anticancer activity.[26-30] These findings provided further impetus to explore the linking of certain non-covalent interacting groups such as dithiocarbamates to a PBD moiety, which has led to the design and synthesis of a variety of PBD hybrids. Considerable interest has been focused on dithiocarbamates which have been found to possess a broad spectrum of biological activities like fungicidal, antibacterial and anticancer activity.[31] Recently, brassnin (**3**, **Fig. 1**), a dithiocarbamate isolated from cabbage, was reported as cancer immunosuppressant and its structural modification has lead to the design and synthesis of sulforamate (**4**, **Fig. 1**).[31-34]

Figure 1. Representative structures of PBD hybrids (1, 5a–c, and 6a,b), dithiocarbamates (990207; 2, brassinin; 3 and sulforamate; 4).

Recently, bi-functional DNA-interacting agents comprising of two types of antitumor agents joined by a linker have attracted considerable attention as a new class of antitumor agents. In this strategy, the coupling of a DNA-groove binder with a DNA-intercalator or the coupling of two DNA-groove-binders provides a basis for modulating the sequence-selective binding behavior and/or tailoring the PBD-hybrid ligands for mixed-sequence recognition. Based on the potent antitumor activity exhibited by the sulphonate[35] and phosphonate substituted PBD[27] hybrids, it was considered of interest to design and synthesize new PBD conjugates by linking dithiocarbamates and piperazine moieties at the C8-position of the PBD with varying alkane spacers. The present paper is mostly focused on the DNA-binding affinity and anticancer activity of the newly synthesized PBD conjugates with an objective to improve the DNA sequence specificity.



Scheme 1. . (i) K_3PO_4, acetone, r.t., 2 h; (ii) CF_3COOH, $CHCl_3$, rt, 8 h.

2. Results and discussion

2.1. Chemistry

The synthetic routes for the preparation of the target conjugates **5a–c** and **6a**, **b** were described in **Scheme 2**. The precursors 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate (**9**)[32] and (2*S*)-*N*-[4-(bromoalkoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**10a–c**) have been prepared by literature methods. The treatment of 3-cyano-3,3-diphenyl propylbromide (**7**) with equimolar amount of *N*-boc piperazine in the presence of CS_2 lead to the formation of conjugate **8**, which was deprotected with TFA to give the intermediate 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate (**9**) as shown in **Scheme 1**.

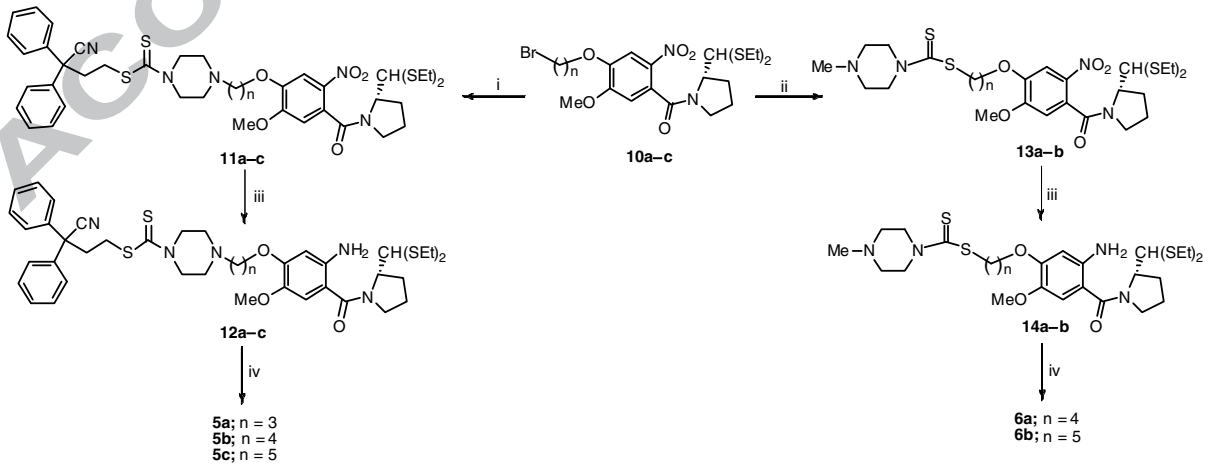
Synthesis of the compounds **5a–c** and **6a, b** has been achieved by employing (2*S*)-*N*-[4-(bromoalkoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethylthioacetals (**10a–c)** as the starting material (**Scheme 2**). Reaction of (2*S*)-*N*-[4-(bromoalkoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethylthioacetals (**10a–c)** with 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate (**9**) gave C8-linked 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate-nitrothioacetals (**11a–c**). The nitro group of these conjugates has been efficiently reduced by employing $SnCl_2.2H_2O$ to afford the corresponding aminothioacetals **12a–c**. Deprotection of the thioacetal group with $HgCl_2$-$CaCO_3$ yielded the desired 3-cyano-3,3-diphenylpropyl 1-piperazinecarbodithioate-pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) conjugates (**5a–c**, **Scheme 2**).

Similarly, nitrothioacetal intermediates **13a**,**b** have been synthesized by reacting **10a–c** with *N*-methylpiperazine in the presence of CS_2 and anhydrous potassium phosphate. Further, the reduction of **13a,b** with $SnCl_2.2H_2O$ has provided aminothioacetals **14a, b**. Deprotective cyclization of these aminothioacetals by using $HgCl_2$-$CaCO_3$ resulted in the formation of the target imine conjugates **6a**,**b** as shown in **Scheme 2**.

2.2. DNA binding studies

The DNA-binding affinity of these PBD conjugates **5a–c** and **6a**,**b** has been investigated by thermal denaturation study of calf thymus (CT) DNA. Binding of molecules into the DNA double helix leads to increase in the helix melting temperature (ΔT_m), the temperature at which the double helix denatures into single stranded DNA. It has been carried out with DNA/ligand molar ratios of 5:1. The increase in the helix melting temperature of DNA (ΔT_m) for each conjugate has been examined at 0 h and 18 h of incubation at 37 ^oC and data is presented in **Table 1**. From this data, it was observed that all these conjugates have shown higher ΔT_m with respect to naturally occurring DC-81 (**1**). Conjugates **5a–c** and **6a** were found to exhibit moderate DNA binding affinity, elevating the helix melting temperature of CT-DNA by 4.0, 2.1, 2.1 and 2.2 ^oC, respectively, after incubation at 37 ^oC for 18 h. *N*-Methyl piperazine-PBD conjugate with dithiocarbamate **6b** has been found to be efficient in stabilizing the double stranded CT-DNA (ΔT_m = 10.9 ^oC). These results demonstrate that such PBD conjugates with piperazine and dithiocarbamate side chain have significant DNA binding affinity as compared to naturally occurring DC-81.

The SAR has been explained based on DNA-binding studies; alkyl spacer can enhance the hydrophobic interactions and might also achieve a superior isohelical fit within the minor groove of DNA. The conjugate **6b** has shown the best DNA-binding affinity probably due to the flexible five carbon chain linker, and absence of bulky end groups, which enables it to rotate and fit in the minor



Scheme 2. (i) K_2CO_3, dry DMF, 48 h; (ii) N-methyl piperazine, CS_2, K_3PO_4, acetone, rt. 2 h; (iii) $SnCl_2.2H_2O$, MeOH, reflux, 2 h; (iv) $HgCl_2$, $CaCO_3$, CH_3CN-H_2O, (4:1), 12 h, 55–58%.

groove without unfavorable close contacts. Hence, longer the alkyl spacer, higher the DNA-binding potential has been observed.

Table 1. Thermal denaturation data for PBD hybrids with piperazine and dithiocarbamate side chains using calf thymus (CT) DNA

Conjugate	Induced ΔT_m (°C)[b] values after incubation at 37 °C for	
	0 h	18 h
5a	2.0	4.0
5b	2.0	2.1
5c	2.0	2.1
6a	2.1	2.2
6b	**6.0**	**10.9**
DC-81 (**1**)	0.3	0.7

[a]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].

[b]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.

2.3. *MTT assay*

Amongst these PBD conjugates, compounds **5a** and **5b** have been selected by the National Cancer Institute (NCI) in a panel of disease-oriented human cancer cell line assay to investigate their cytotoxic potential. According to the screening data, conjugate **5a** was found to be potentially cytotoxic against the nine cell panels with GI_{50} values of <0.99 μM, as shown in **Table 2**. Relatively higher sensitivity to the conjugates described here was found for cell lines of melanoma (LOX IMVI, M14), leukemia (MOLT-4, RPMI-8226, SR), CNS (SF-268, U251), ovarian (IGROV1, OVCAR-3), breast (MCF-7) and renal cancer (786-0, ACHN, CAKI-1, UO-31).

Moreover, the PBD conjugates **5c** and **6a**,**b** have also been evaluated for their *in vitro* cytotoxicity in some selected human cancer cell lines like MCF7 (breast), A2780 (ovarian), Colo205 (colon), PC3 (prostate), SiHa (cervix), A-549, Hop62 (lung) and KB (leukemia), by employing the sulforhodamine B (SRB) assay. The results displayed in **Table 3** show that **5c** and **6a**,**b** were also significantly cytotoxic, with GI_{50} values (the molar concentration of the drug that inhibits 50% net cell growth inhibition) ranging from 0.10 to 1.7 μM.

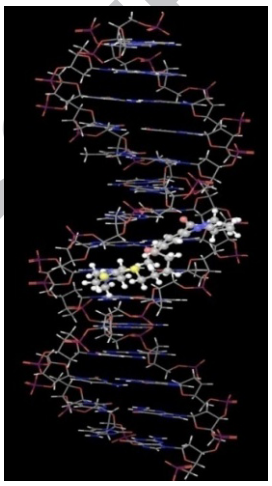


Figure 2. Docked pose of **6b** in the minor groove of DNA 5′-GGGGCGAGAGAGGGG-3′.

Table 2. *In vitro* anticancer data for selected conjugates **5a** and **5b**[a]

Panel cell lines	Response parameter GI_{50}[b] (μM) for conjugate		Panel cell lines	Response parameter GI_{50}[b] (μM) for conjugate	
	5a	**5b**		**5a**	**5b**
Leukemia			*Ovarian cancer*		
CCRF-CEM	-	**0.53**	IGROV1	**0.47**	**0.42**
HL-60 (TB)	3.06	**0.32**	OVCAR-3	**0.83**	1.20
K-562	1.22	3.29	OVCAR-4	2.19	1.82
MOLT-4	**0.63**	**0.35**	OVCAR-5	1.14	2.47
RPMI-8226	**0.79**	**0.49**	OVCAR-8	1.71	1.59
SR	**0.99**	**0.25**	SK-OV-3	2.35	2.96
Non-small cell lung cancer			*Renal cancer*		
A549/ATCC	1.28	1.61	786-0	**0.23**	1.28
EKVX	2.06	1.88	A498	1.10	1.19
HOP-62	**0.40**	**0.71**	ACHN	**0.81**	1.16
HOP-92	**0.54**	1.07	CAKI-1	**0.35**	5.97
NCI-H226	1.52	1.26	SN12C	1.04	**0.41**
NCI-H23	**0.81**	1.68	TK-10	1.83	1.16
NCI-H322M	2.34	1.40	UO-31	**0.55**	1.26
NCI-H460	1.03	1.25			**0.59**
NCI-H522	1.59	**0.19**			
Colon cancer			*Breast cancer*		
COLO 205	1.02	5.66	MCF7	**0.54**	**0.43**
HCC-2998	1.39	3.41	NCI/ADR-RES	3.77	3.05
HCT-116	**0.36**	-		1.23	1.53
HCT-15	1.56	3.90	MDA-MB-231/ATCC	1.68	**0.72**
HT29	1.02	>100		1.64	1.04
KM12	2.52	3.78	HS-578T	1.23	1.42
SW-620	1.10	-	MDA-MB-435	4.36	**0.57**
				-	**0.49**
			BT-549		
			T-47D		
			MDA-MB-468		
CNS cancer			*Prostate cancer*		
SF-268	**0.53**	1.23	PC-3	1.45	**0.69**
SF-295	1.11	-	DU-145	**0.82**	1.47
SF-539	1.46	1.69			
NB-19	3.89	1.67			
SNB-75	1.36	1.64			
U251	**0.41**	1.11			
Melanoma					
LOX IMVI	**0.86**	2.58			
MALME-3M	1.28	3.04			
M14	**0.90**	3.04			
SK-MEL-2	1.01	2.35			
SK-MEL-28	1.80	3.96			
SK-MEL-5	1.46	2.82			
UACC-257	1.98	4.17			
UACC-62	1.20	3.55			

[a]Data obtained from the NCI's in vitro disease-oriented human tumor cell screen [34–36]. [b]The responses parameter: GI_{50} is interpolated values representing the molar concentrations at which percentage growth is +50.

Table 3. Cytotoxicity (GI_{50} μM) and (LC_{50} μM)[a] of conjugates **5c**, **6a**,**b** and adriamycin (ADR) against eight human cancer cell lines

Cell lines	**5c**		**6a**		**6b**		**ADR**	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}	GI_{50}	LC_{50}	GI_{50}	LC_{50}
MCF7	2.56	3.66	**0.15**	2.4	**0.13**	2.2	$<10E^{-7}$	0.17
A2780	n.d	n.d	**0.16**	>100	**0.14**	28	0.002	$<10E^{-7}$
Colo 205	2.52	6.08	**0.14**	>100	**0.12**	>100	14.7	>100
PC-3	2.01	>100	**0.15**	>100	**0.12**	26	0.23	$<10E^{-7}$
SiHa	n.d	n.d	1.7	>100	**0.15**	>100	1.9	>100
A-549	3.69	>100	**0.16**	>100	**0.14**	>100	13	>100
Hop-62	2.46	>100	**0.18**	>100	**0.10**	>100	$<10E^{-7}$	$<10E^{-7}$
KB	n.d	n.d	**0.16**	>100	**0.14**	30	0.16	>100

[a]Response parameter; n.d: Not determined.

2.4. Molecular Modelling

In order to further substantiate the biological activity of these conjugates, molecular modelling studies were performed. Seven different B-DNA duplexes with varying sequences have been constructed and investigated for the interaction with the ligands, among which five DNA duplexes containing triplet AGA (Pu-G-Pu), the preferred binding site for PBD, were found to be the most favored sequences for docking. Among these five sequences, docking results for the 15-mer sequence 5′-GGGGCGAGAGAGGGG-3′ containing the central AGA has shown excellent correlation with DNA-binding studies and has been employed for further studies. Molecular docking simulations carried out using Glide[36] with default settings show that these molecules prefer the minor groove over the major groove and intercalation as well as the binding is based on non-bonded
interactions like hydrogen-bonding, Vander Waals contacts, hydrophobic and electrostatic effects in addition to the covalent linkage formed between the imine of PBD moiety and exocyclic C2-amino group of the guanine. One of the representative conjugates **6b** has shown the best score probably due to flexible five carbon chain linker, and absence of bulky end groups, which enables it to rotate and fit in the minor groove without unfavorable close contacts (**Fig. 2**). The best scored poses of DNA-ligand complexes from the docking studies have been used for molecular dynamic simulations. The results of molecular docking studies are highly consistent with the cytotoxicity as well as thermal denaturation data.

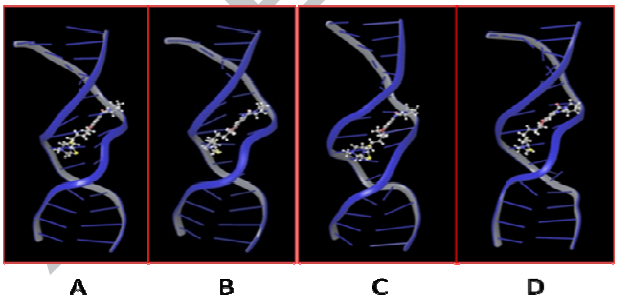


Figure 3. Snapshots of DNA-**6b** complex at 0 ns (A), 1 ns (**B**), 2 ns (**C**) and 4 ns (**D**) time scale.

Molecular dynamics (MD) simulations were performed to provide some insight into the dynamic nature of the complexes and assess the effect of temperature and solvent. The simulation time scale was 5 ns and 500 snapshots were taken, the final binding interaction energies were calculated as the average of all the 500 snapshots. It was clear from the RMSDs that the docked conformation of compound **6b** was stable till 5 ns MD simulation and the docked poses of all the remaining compounds were reasonably stable up to 3 ns MD simulation. The snapshots of this MD run confirmed the strong binding ability of the compound **6b** to DNA minor-groove as shown in **Figure 3**.

The energy of interaction (E_{int}) between the DNA and the PBD conjugates in a complex was calculated as a measure of stability of that complex as shown in **Table 4**. The complex formed by **6b** is energetically more stable than the complex formed by **6a** because of longer linker unit in **6b** and more stable than **5a**–**c** due to absence of bulky end group which enables better non-bonded interactions due to perfect isohelical fit in the minor groove. It was very clear from the high negative interaction energies that all the docked complexes are favorable and stable.

Table 4. Energy of interaction (E_{int}) calculated for DNA-PBD conjugate complexes by generalized Born method after 5 ns MD simulation.

Complex	$E_{int\ (kcal\ mol^{-1})}$
DNA-5a	-111.23
DNA-5b	-101.86
DNA-5c	-91.01
DNA-6a	-105.30
DNA-6b	-114.56

3. Conclusion

In conclusion, we have designed and synthesized a new series of dithiocarbamate bridged piperazine-pyrrolobenzodiazepine conjugates. These molecules have exhibited moderate to significant DNA-binding ability. Introduction of *N*-methylpiperazinedithiocarbamate with five-membered alkane spacer to the PBD increased the DNA-binding activity considerably in one of the PBD derivative **6b** (ΔT_m = 10.9 °C). Compound **5a** has displayed cytotoxic potency against many cell lines and exhibits a wide spectrum of activity against 33 cell lines in nine cancer phenotypes with GI_{50} values of < 0.99 μM. Further, detailed molecular modeling studies involving molecular docking, molecular dynamics and MM-PBSA calculations support the biological data. The promising results obtained for these new PBD-dithiocarbomate derivatives presented in this paper, make them possible candidates for the treatment of cancer, and encourage us to advance in the synthesis and evaluation of new PBD derivatives.

The detailed molecular mechanism for these PBD conjugates is being further investigated.

4. Experimental

4.1. General

The majority of the solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenone ketyl), acetone (potassium permanganate), and acetonitrile (phosphorous pentoxide). 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 mesh). 1H and 13C NMR spectra were recorded on INOVA (400 MHz) or Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) spectrometer using tetramethyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) downfield from tetramethyl silane. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants are reported in Hertz (Hz). Low resolution mass spectra were recorded on a VG-7070H Micromass mass spectrometer at 200 oC, 70 eV with trap current of 200 lA, and 4 kV acceleration voltage. FABMS spectra were recorded on LSIMS-VG-AUTOSPEC-Micromass. Melting points were recorded on Electrothermal 9100 and are uncorrected. All computational studies were done with a Red Hat Enterprise Linux version 5.0 using Maestro software version 9.5 (Schrödinger, LLC, New York, NY, 2013).

4.2. Chemistry

***tert*-Butyl 4-(((3-cyano-3,3-diphenylpropyl)thio)carbonothioyl)piperazine-1-carboxylate (8).** To a stirred solution of *N*-boc piperazine (186 mg, 1 mmol) and anhydrous potassium phosphate (425 mg, 2 mmol) in acetone (20 mL) was added carbon disulfide 10 drops (~ 2.5 mmol) drop-wise. After stirring for 30 min, 3-cyano-3,3-diphenylpropyl bromide (**7**; 300 mg, 1 mmol) was added. The stirring was further continued about 2 h, the resulted solid was filtered off and the filtrate was concentrated. The residue was dissolved in ethyl acetate (20 mL), and washed with water. The organic layer was dried over anhydrous Na_2SO_4, evaporated the solvent and the residue was purified by column chromatography using 50% ethyl acetate/hexane to afford compound **8** (384 mg, yield 80%). Cream colour solid; mp: 144–146 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm: 7.45 (d, J = 7.55 Hz, 4H), 7.36 (t, J = 7.55 Hz, 4H), 7.29 (d, J = 6.79 Hz, 2H), 4.22 (bs, 2H), 3.94 (bs, 2H), 3.53 (t, 4H), 3.32–3.41 (m, 2H), 2.74–2.83 (m, 2H), 1.46 (s, 9H); MS (ESI): *m/z* 504 $[M+Na]^+$.

3-Cyano-3,3-diphenylpropyl piperazine-1-carbodithioate (9). A solution of **8** (481 mg, 1 mmol) in dichloromethame (15 mL) was added trifluoroacetic acid (1.14 gm, 10 mmol) at 0 ^{o}C and the mixture was stirred at room temperature for 8 h. The solvent was evaporated and the solution was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with chloroform (2 x 15 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the compound **9** (285 mg, 75%). Thick liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm: 7.46 (d, J = 8.0 Hz, 4H), 7.38 (dd, J = 5.50 Hz, 1.9 Hz, 4H), 7.30 (t, 2H), 4.29 (bs, 2H), 3.87 (bs, 2H), 3.39 (m, 2H), 2.93 (bs, 4H), 2.83 (m, 2H), 1.72 (bs, 1H); MS (ESI): *m/z* 382.4 $[M+H]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(3-(4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxy-5-nitrophenoxy)propyl)piperazine-1-carbodithioate (11a). To a solution of **10a** (521 mg, 1 mmol) in dry DMF (10 mL) was added anhydrous K_2CO_3 (552 mg, 4 mmol) and compound **9** (381 mg, 1 mmol). The reaction mixture was stirred at room temperature for 48 h. The K_2CO_3 was removed by suction filtration and the solvent was evaporated under vacuum. The crude product thus obtained was purified by column chromatography using 60% ethyl acetate-hexane to afford title compound **11a** (657 mg, 80%). Light yellow solid; mp: 124–126 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δppm: 7.71 (s, 1H), 7.29–7.57 (m, 10H), 6.83 (s, 1H), 4.88 (d, J = 3.39 Hz, 1H), 4.65–4.76 (m, 1H), 4.31 (t, J = 6.79 Hz, 2H), 4.18 (t, J = 6.04 Hz, 2H), 3.94 (s, 3H), 3.33–3.43 (m, 2H), 3.17–3.32 (m, 2H), 2.65–2.88 (m, 4H), 2.48–2.63 (m, 8H), 2.21–2.37 (m, 2H), 2.02–2.18 (m, 2H), 1.61–1.86 (m, 4H), 1.16–1.47 (m, 6H); MS (ESI): *m/z* 822 $[M]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(4-(4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxy-5-nitrophenoxy)butyl)piperazine-1-carbodithioate (11b). The compound **11b** was prepared following the method described for the compound **11a**, employing **10b** (535 mg, 1 mmol) and **9** (381 mg, 1 mmol), and the crude product was purified by column chromatography (60% ethyl acetate-hexane) to afford the compound **11b** (668 mg, 80%). Light yellow solid; mp: 132–134 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δppm: 7.67 (s, 1H), 7.25–7.50 (m, 10H), 6.83 (s, 1H), 4.88 (d, J = 3.77 Hz, 1H), 4.61–4.76 (m, 1H), 4.31 (t, J = 6.79 Hz, 2H), 4.13 (t, J = 6.79 Hz, 2H), 3.95 (s, 3H), 3.33–3.43 (m, 2H), 3.17–3.32 (m, 2H), 2.67–2.87 (m, 4H), 2.41–2.60 (m, 8H), 2.21–2.37 (m, 2H), 2.04–2.19 (m, 2H), 1.88–2.03 (m, 2H), 1.53–1.86 (m, 4H), 1.21–1.40 (m, 6H); MS (ESI): *m/z* 836 $[M]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(5-(4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxy-5-nitrophenoxy)pentyl)piperazine-1-carbodithioate (11c). The compound **11c** was prepared following the method described for the compound **11a**, employing **10c** (549 mg, 1 mmol) and **9** (381 mg, 1 mmol), and the crude product was purified by column chromatography (60% ethyl acetate-hexane) to afford the compound **11c** (680 mg, 80%). Light yellow solid; mp: 128–130 ºC; ^{1}H NMR (200 MHz, $CDCl_3$): δppm: 7.66 (s, 1H), 7.23–7.50 (m, 10H), 6.82 (s, 1H), 4.88 (d, J = 3.77 Hz, 1H), 4.67–4.77 (m, 1H), 4.31 (t, J = 6.79 Hz, 2H), 4.09 (t, J = 5.85 Hz, 2H), 3.94 (s, 3H), 3.33–3.44 (m, 2H), 3.17–3.32 (m, 2H), 2.65–2.90 (m, 4H), 2.48–2.62 (m, 8H), 2.38–2.47 (m, 2H), 2.22–2.37 (m, 2H), 2.00–2.19 (m, 2H), 1.86–1.98 (m, 2H), 1.48–1.77 (m, 4H), 1.22–1.41 (m, 6H); MS (ESI): *m/z* 850 $[M]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(3-(5-amino-4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxyphenoxy)propyl)piperazine-1-carbodithioate (12a). The stirred mixture of **11a** (822 mg, 1 mmol) and $SnCl_2.2H_2O$ (1.125 g, 5 mmol) in methanol (20 mL) was refluxed for 5 h or until the TLC indicated that reaction was completed. The methanol was evaporated by vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate and chloroform (2 x 30 ml and 2 x 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the crude amino diethyl thioacetal **12a** (617 mg, 78%) as yellow oil, which was used directly for the next step.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(4-(5-amino-4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-

methoxyphenoxy)butyl)piperazine-1-carbodithioate (12b). The compound **12b** was prepared following the method described for the compound **12a**, employing the **11b** (836 mg, 1 mmol) and $SnCl_2.2H_2O$ (1.125 g, 5 mmol) to afford the amino diethyl thioacetal **12b** as a yellow oil (628 mg, 78%), which was used directly for the next step without further purification.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(5-(5-amino-4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxyphenoxy)pentyl)piperazine-1-carbodithioate (12c). The compound **12c** was prepared following the method described for the compound **12a**, employing the **11c** (850 mg, 1 mmol) and $SnCl_2.2H_2O$ (1.125 g, 5 mmol) to afford the amino diethyl thioacetal **12c** as a yellow oil (639 mg, 78%), which was used directly for the next step without further purification.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(3-((7-methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)propyl)piperazine-1-carbodithioate (5a). A solution of **12a** (792 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in acetonitrile:water (4:1) was stirred slowly at room temperature until TLC indicates complete loss of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) filtered through a celite pad. The clear organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 ml), brine (20 ml) and the combined organic phase is dried (Na_2SO_4). The organic layer was evaporated in vacuum and purified by column chromatography (95% CH_2Cl_2-MeOH) to give compound **5a** (366 mg, 55% yield) as cream yellow solid. This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. mp: 114–116 ^oC; $[\alpha]_D^{25}$ = +0.26 (*c* = 0.9 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δppm: 7.64 (d, *J* = 3.82 Hz, 1H), 7.28–7.55 (m, 10H), 6.80 (s, 1H), 6.36 (s, 1H), 3.96–4.23 (m, 4H), 3.95 (s, 3H), 3.44–3.86 (m, 4H), 3.25–3.43 (m, 2H), 2.73–2.91 (m, 2H), 2.20–2.62 (m, 7H), 1.38–2.15 (m, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 197.1, 164.8, 162.9, 152.1, 149.7, 143.7, 140.7, 139.7, 128.9, 127.4, 119.2, 118.5, 117.8, 112.8, 79.4, 59.3, 56.9, 56.6, 54.7, 53.6, 51.1, 44.1, 38.7, 29.3, 28.9, 28.1, 24.1; HRMS (ESI): *m/z* calcd for $C_{37}H_{41}N_5O_3S_2$ 668.2691, found 668.2699 $[M+H]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(4-((7-methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)butyl)piperazine-1-carbodithioate (5b). The compound **5b** was prepared following the method described for the preparation of the compound **5a**, employing **12b** (806 mg, 1 mmol) and $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to afford the compound **5b** as a pale yellow solid (388 mg, 57%). mp: 100–102 ^oC; $[\alpha]_D^{25}$ = +5.4 (*c* = 1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δppm: 7.67 (d, *J* = 4.71 Hz, 1H), 7.29–7.57 (m, 10H), 6.81 (s, 1H), 6.34 (s, 1H), 3.97–4.44 (m, 4H), 3.94 (s, 3H), 3.45–3.80 (m, 4H), 3.30–3.46 (m, 2H), 2.74–2.91 (m, 2H), 2.23–2.65 (m, 8H), 1.35–2.20 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 196.9, 164.6, 163.1, 152.4, 149.6, 143.5, 141.1, 139.6, 129.2, 127.4, 119.1, 118.5, 117.6, 112.8, 79.2, 59.1, 57.1, 56.6, 54.6, 53.6, 51.4, 44.2, 38.7, 29.4, 28.9, 28.1, 24.8, 24.2; HRMS (ESI): *m/z* calcd for $C_{38}H_{43}N_5O_3S_2$ 682.2847, found 682.2853 $[M+H]^+$.

(*S*)-3-Cyano-3,3-diphenylpropyl 4-(5-((7-methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1H-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)pentyl)piperazine-1-carbodithioate (5c). The compound **5c** was prepared following the method described for the preparation of the compound **5a**, employing **12c** (820 mg, 1 mmol), to afford the compound **5c** as a pale yellow solid (382 mg, 55%). mp: 110–112 ^oC; $[\alpha]_D^{25}$ = +0.44 (*c* = 0.7 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δppm: 7.67 (d, *J* = 4.71 Hz, 1H), 7.29–7.54 (m, 10H), 6.79 (s, 1H), 3.96–4.18 (m, 4H), 3.95 (s, 3H), 3.49–3.88 (m, 4H), 3.25–3.45 (m, 2H), 2.73–2.91 (m, 2H), 2.22–2.63 (m, 8H), 1.38–2.17 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 196.1, 164.9, 163.3, 152.3, 148.9, 143.7, 140.1, 129.4, 128.1, 126.2, 117.4, 116.1, 109.9, 107.8, 69.9, 56.8, 56.1, 54.2, 53.1, 52.4, 49.9, 39.7, 38.4, 29.7, 28.6, 24.7, 23.7; HRMS (ESI): *m/z* calcd for $C_{39}H_{45}N_5O_3S_2$ 696.3003, found 696.3011 $[M+H]^+$.

(*S*)-4-(4-(2-(Bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxy-5-nitrophenoxy)butyl 4-methylpiperazine-1-carbodithioate (13a). To a solution of 1-methylpiperazine (100 mg, 1 mmol) in dry acetone (10 mL) was added carbon disulfide 10 drops (~2.5 mmol) and anhydrous K_3PO_4 (425 mg, 2 mmol). The mixture was stirred <10 ^oC for 1 h. Then **10b** (535 mg, 1 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. TLC using ethylacetate as a solvent system monitored the reaction. The potassium phosphate was removed by suction filtration and the solvent was removed under vacuum. The crude product was purified by column chromatography using 40% ethyl acetate-hexane to afford pure compound **13a** (504 mg, 80%). Light yellow solid; mp: 80–82 ºC; ^1H NMR (200 MHz, $CDCl_3$) δppm: 7.64 (s, 1H), 6.77 (s, 1H), 4.83 (d, J = 4.15 Hz, 1H), 4.61–4.76 (m, 1H), 4.14 (t, *J* = 5.81, 6.12 Hz, 2H), 3.95 (s, 3H), 3.39 (t, *J* = 6.64, 5.83 Hz, 2H), 3.17–3.30 (m, 2H), 2.51–2.90 (m, *J* = 4.98 Hz, 14H), 2.33 (s, 3H), 1.87–2.12 (m, 6H), 1.23–1.42 (q, 6H); MS (ESI): *m/z* 631 $[M]^+$.

(*S*)-5-(4-(2-(Bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxy-5-nitrophenoxy)pentyl 4-methylpiperazine-1-carbodithioate (13b). To a solution of 1-methylpiperazine (100 mg, 1 mmol) in dry acetone (10 mL) was added carbon disulfide 10 drops (~2.5 mmol) and anhydrous K_3PO_4 (425 mg, 2 mmol). The mixture was stirred <10 °C for 1 h. Then **10c** (549 mg, 1 mmol). The reaction mixture was stirred at room temperature for 12 h. TLC using ethylacetate as a solvent system monitored the reaction. The potassium phosphate was removed by suction filtration and the solvent was removed under vacuum. The crude product was purified by column chromatography using 40% ethyl acetate-hexane to afford pure compound of **13b** (528 mg, 82%). Light yellow solid; mp: 86–88 ºC; ^1H NMR (200 MHz, $CDCl_3$) δ ppm: 7.67 (s, 1H), 6.83 (s, 1H), 4.88 (d, 1H), 4.65–4.78 (m, 1H), 4.11 (t, *J* = 7.03 Hz, 2H), 3.96 (s, 3H), 3.36 (t, *J* = 7.03 Hz, 2H), 3.21–3.30 (m, 2H), 2.75–2.88 (m, 4H), 2.52 (t, *J* = 5.47 Hz, 4H), 2.35 (s, 3H), 2.17–2.30 (m, 2H), 2.12 (t, *J* = 7.81 Hz, 2H), 2.00–2.07 (m, 2H), 1.94 (t, *J* = 7.81 Hz, 2H), 1.75–1.88 (m, 2H), 1.67 (t, *J* = 3.90 Hz, 2H), 1.57–1.65 (m, 2H), 1.16–1.44 (m, 6H); MS (ESI): *m/z* 645 $[M]^+$.

(*S*)-4-(5-Amino-4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxyphenoxy)butyl 4-methylpiperazine-1-carbodithioate (14a). The compound **13a** (631 mg, 1 mmol) dissolved in methanol (20 mL) and added $SnCl_2.2H_2O$ (1.125 g, 5 mmol) was refluxed for 5 h. The methanol was evaporated by vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate and chloroform (2 x 30 ml and 2 x 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the crude amino diethyl thioacetal **14a** (468 mg, 78%) as yellow oil, which was used directly in the next step without further purification.

(*S*)-5-(5-Amino-4-(2-(bis(ethylthio)methyl)pyrrolidine-1-carbonyl)-2-methoxyphenoxy)pentyl 4-methylpiperazine-1-carbodithioate (14b). The compound **14b** was prepared

following the method described for the compound **14a**, employing the compound **13b** (645 mg, 1 mmol) to afford the amino diethyl thioacetal **14b** as a yellow oil (479 mg, 78%), which was used directly in the next step.

(*S*)-4-((7-Methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)butyl 4-methylpiperazine-1-carbodithioate (6a). A solution of **14a** (601 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in acetonitrile:water (4:1) was stirred slowly at room temperature for overnight. The reaction mixture was diluted with ethyl acetate (30 mL) filtered through a celite pad. The clear organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried over (Na_2SO_4). The organic layer was evaporated under vacuum and purified by column chromatography using 4% MeOH-$CHCl_3$ to give compound **6a** (276 mg, 58%) as light yellow solid. This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. mp: 94–96 ^oC; ^1H NMR (400 MHz, $CDCl_3$) δppm: 7.61 ppm (d, J = 4.68 Hz, 1H), 7.45 (s, 1H), 6.73(s, 1H), 3.80–4.07 (m, 4H), 3.79 (s, 3H), 3.61–3.74 (m, 2H), 3.3 (t, J = 7.03 Hz, 4H), 2.44 (t, J = 5.46 Hz, 2H), 2.27 (s, 3H), 1.77–2.05 (m, 4H), 1.51–1.54 (m, 1H), 1.36 (d, J = 5.46 Hz, 1H), 1.06–1.25 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 196.9, 164.5, 163.5, 152.3, 148.9, 140.2, 117.1, 108.1, 107.2, 79.5, 57.1, 54.7, 52.8, 51.7, 47.2, 36.8, 31.1, 28.1, 26.7, 24.6; HRMS (ESI): *m/z* calcd for $C_{23}H_{32}N_4O_3S_2$ 477.1952, found 477.1961 $[M+H]^+$.

(*S*)-5-((7-Methoxy-5-oxo-2,3,5,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-8-yl)oxy)pentyl 4-methylpiperazine-1-carbodithioate (6b). A solution of **14b** (615 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in acetonitrile:water (4:1) was stirred slowly at room temperature for overnight. The reaction mixture was diluted with ethyl acetate (30 mL) filtered through a celite pad. The clear organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried (Na_2SO_4). The organic layer was evaporated under vacuum and purified by column chromatography using 4% MeOH-$CHCl_3$ to give compound **6b** (284 mg, 58%) as light yellow solid. This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. mp: 90–92 ^oC; ^1H NMR (400 MHz, $CDCl_3$) δppm: 7.62 (d, J = 3.90 Hz, 1H), 7.22 (s, 1H), 6.75 (s, 1H), 3.92–4.11 (m, 4H), 3.90 (s, 3H), 3.41–3.85 (m, 3H), 3.28 (t, J = 7.03 Hz, 2H), 2.45 (m, 8H), 2.29 (s, 3H), 1.41–2.18 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 197.1, 164.7, 163.6, 152.4, 149.0, 140.4, 117.2, 108.3, 107.1, 79.0, 56.9, 54.6, 52.7, 50.9, 50.2, 46.9, 36.6, 31.4, 30.08, 29.7, 26.6, 23.9; HRMS (ESI): *m/z* calcd for $C_{24}H_{34}N_4O_3S_2$ 491.2111, found 491.2119 $[M+H]^+$.

4.3. DNA binding studies

Compounds were subjected to thermal denaturation studies with duplex-form CT-DNA using reported method. Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_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 DMSO to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions were incubated at 37 ^oC for 0 and 18 h prior to analysis. Samples were monitored at 260 nm using a Beckman–Coulter DU 800 spectrophotometer fitted with high performance temperature controller, and heating was applied at 1 ^oC min ^{1} in the range of 40–90 ^oC. DNA helix→coil transition temperatures I were obtained from the maxima in the d(A260)/dT derivative plots. Results are given as means ± standard deviation from three determinations and are corrected for the effects of DMSO co-solvent using a linear correction term. Drug-induced alterations in DNA melting behaviour 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 69.2 ± 0.01 ^oC. The fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

4.4. MTT assay

In-routine compounds **5c**, **6a** and **6b** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines of MCF7 (breast), A2780 (ovarian), Colo205 (colon), PC3 (prostate), SiHa (cervix), A-549, Hop62 (lung) and KB (Leukemia) origin. 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.[38,39]

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 ^oC, 5% CO_2, 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. Plates were incubated further for 48 h and assay was terminated by the addition of 50 lL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and incubated for 60 min at 4 ^oC.

The plates were washed five times with tap water and air-dried. 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 air-dried. 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 wavelength. Percent growth was calculated on a plate–byplate basis for test wells relative to control wells.

Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) x 100.

4.5. Molecular modelling Studies

The hybrids were built and prepared using Ligprep 2.7 and geometrically minimized with Macromodel 10.1 followed by conformational analysis in Maestro 9.5. Truncated Newton Conjugate Gradient (TNCG) minimization method was used with 500 iterations and convergence threshold of 0.05 kJ/mol. All the DNA duplexes were prepared using protein preparation wizard. The Glide XP 6.5 algorithm was employed for docking. The lowest energy pose for each compound was further subjected to molecular dynamics simulations using Desmond 3.5 with OPLS-AA force field in explicit solvent with the TIP3P water model. Before MD simulations, the system was pre-equilibrated using Constant-Volume (NVT) MD simulation for the first 100 ps during which temperature of the system was raised from 0 to 300 K and for further simulation the temperature was maintained at 300 K. Subsequently, the system was equilibrated in NPT which is composed of minimization and short MD simulation (12 and 24 ps) to relax the model system. After that, long equilibration MD simulation was performed for first 2 ns and long production

MD simulation for 5 ns. Data were collected every 10 ps during the MD runs. The complexes present in trajectory file after production phase of MD simulations, were clustered according to the RMSD of backbone. The interaction energies in these clusters were calculated using the using MM/ GBSA present in Prime 3.3.

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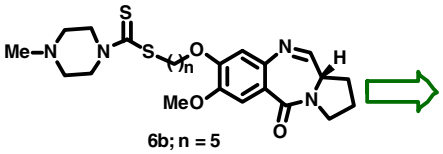
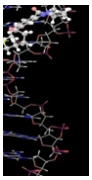
Graphical Abstract



6b; n = 5

In vitro 0.10–0.14 μM (GI_{50})

ΔT_m = 10.9 °C at 37 °C in 18 h



Research Highlights

- C8-linked dithiocarbamate/piperazine-PBD conjugates (**5a–c**, **6a,b**) were synthesized.
- The conjugates **5a** and **5b** have been screened for their cytotoxicity by NCI.
- The compounds **5c** and **6a**,**b** have been also exhibited significant *in vitro* cytotoxicity.
- Molecular modeling studies predict that **6b** exhibits superior than other compounds.