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Bioorganic & Medicinal Chemistry 14 (2006) 385–394
Bioorganic & Medicinal Chemistry

DNA binding potential and cytotoxicity of newly designed pyrrolobenzodiazepine dimers linked through a piperazine side-armed-alkane spacer☆

Ahmed Kamal,* P. S. Murali Mohan Reddy, D. Rajasekhar Reddy and E. Laxman

Biotransformation Laboratory, Division of Organic Chemistry, Indian Institute of Chemical Technology, Hyderabad 500 007, India

Received 16 June 2005; revised 9 August 2005; accepted 9 August 2005
Available online 26 September 2005

Abstract—New pyrrolobenzodiazepine (PBD) dimers have been developed that are composed of two DC-81 subunits tethered to their C8 positions through piperazine moiety side-armed with alkaneoxy linkers (composed of 2–5 carbons). DNA thermal denaturation studies show that after 18 h of incubation with calf thymus DNA at a 1:5 ligand/DNA ratio, one of them, **6a**, increases the ΔT_m value by 24.0 °C. Thus, incorporation of a piperazine moiety instead of an inert alkanedioxy linker alone significantly enhances the DNA binding ability, and the analogous dimer **4** that lacks a piperazine moiety in the linker spacer elevates melting by only 15.1 °C under identical experimental conditions. This illustrates the effect of introducing a piperazine ring in the middle of such an alkanedioxy linker which produces several hydrophobic interactions and could also achieve a superior isohelical fit within the DNA minor groove. Interestingly, these dimers **6a–d** are significantly more cytotoxic than **4** in a number of human cancer cell lines, in particular, compound **6c** is highly potent for almost all the nine human cancer cell lines.

1. Introduction

There have been considerable attempts with the objective of targeting specific sequences in DNA by synthetic ligands with the idea of designing both drugs and molecular probes for DNA polymorphism compounds that bind in the minor groove of DNA.[2–4] These classes of DNA ligands possess several biological activities particularly antiviral and antitumor activity.[5] One of the class of compounds is able to induce permanent DNA damage through direct or indirect irreversible interaction with DNA nucleotides comprised of antibiotic compounds of natural, synthetic, and semi-synthetic origin. One group of naturally occurring alkylating antitumor antibiotics are pyrrolobenzodiazepines (PBDs). These antitumor antibiotics are a class of sequence selective DNA binding agents derived from *Streptomyces* species,[6] well-known members of which include DC-81 (**1**), tomaymycin (**2**), and anthramycin (**3**) (Fig. 1). The cytotoxicity and antitumor activity of these are attributed to their property of sequence selective covalent binding with the 2-NH_2 group of guanine in the minor groove of duplex DNA via an acid labile aminal bond to the electrophilic imine at the N10-C11 position.[6,7] The N10-C11 carbinolamine bond exists in the equivalent imine of carbinolamine methyl ether form depending on the precise structure of the compound and method of isolation.[8] PBD monomers span three DNA base pairs with the preference for Pu-G-Pu triplets. The PBDs have been shown to interfere with the interaction of endonuclease enzymes with DNA[9] and to block transcription by inhibiting RNA polymerase in a sequence-specific manner.[10] Thurston and co-workers reported the first C8/C8′-linked PBD dimer (DSB-120) (**4**) in which two DC-81 subunits are joined through their aromatic A-ring at phenol positions by an inert propyldioxy linkage.[11,12] These C8-diether-linked PBD dimers exhibit enhanced DNA binding affinity compared to the monomer DC-81 and span six DNA base pairs actively recognizing a 5′-GATC sequence, that is, doubling of the DNA binding size.[13] However, these lack in vivo antitumor activity probably because of the low bioavailability and also due to excessive electrophilicity at the N10-C11 imine moiety.[14] Subsequently, a PBD dimer (SJG-136) (**5**) containing C2/C2′-exomethylene functionalities was designed to reduce the electrophilicity of the molecule by decreasing the deactivation

Keywords: Pyrrolobenzodiazepine; DNA-binding ability; Cytotoxicity; Piperazine.
☆ See Ref. 1.
* Corresponding author. Tel.: +91 40 27193157; fax: +91 40 27193189; e-mail addresses: ahmedkamal@iict.res.in; ahmedkamal@iictnet.org

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doi:10.1016/j.bmc.2005.08.020

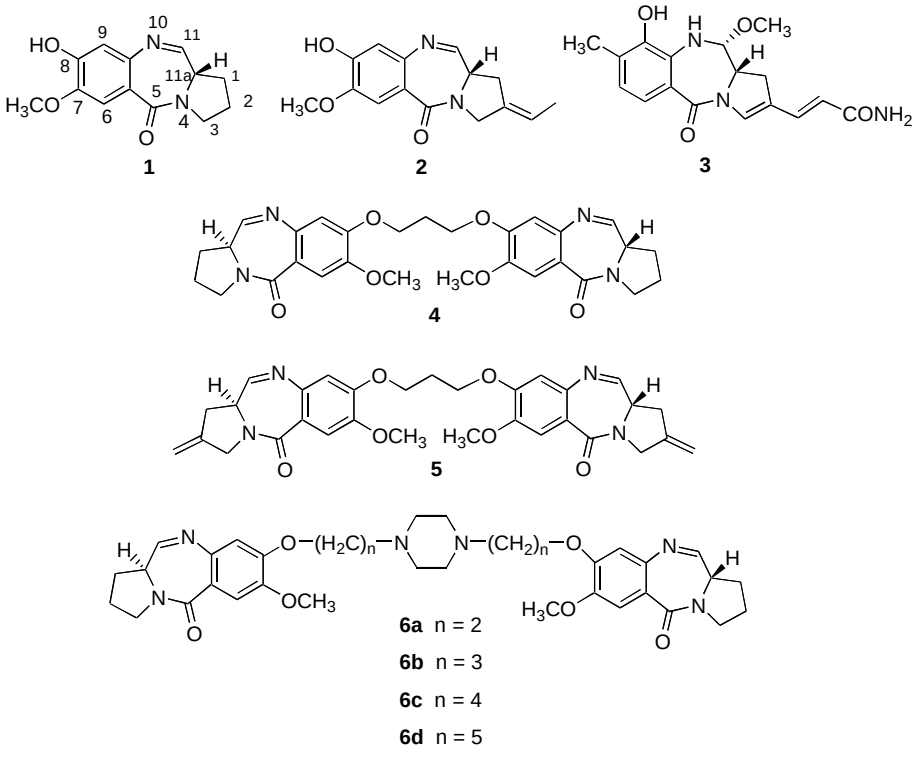


Figure 1. Structures of the PBD monomer DC-81 (**1**), tomaymycin (**2**), anthramycin (**3**), DSB-120 (**4**), SJG-136 (**5**), piperazine-linked PBD dimers (**6a**–**d**).

of cellular nucleophiles.[15] This compound exhibits significant in vivo potential and is under clinical evaluation (Fig. 1). Additionally, in a recent report it has been observed that the length of the linker modulates DNA cross-linking property and cytotoxic potency. Recently, a large number of structurally modified PBDs have been synthesized and evaluated for their biological activity particularly for their DNA binding potential and antitumor property. Interestingly, a number of these compounds have been selected for preclinical studies but unfortunately most of them did not proceed beyond that stage mainly because of problems related to poor bioavailability and low water solubility.[16]

During the last decade, many piperazine derivatives have been synthesized as useful chemotherapeutic agents for various diseases. Bis-1,4-dialkyl-piperazines have been extensively investigated and have been reported as antibacterial[17–19] and antineoplastic agents.[17,20,21] Michejda and co-workers[22] reported symmetrical bifunctional agents as a promising antitumor class of compounds with remarkable selectivity against colon cancers that possess a piperazine moiety in its linker spacer. Recently, soluble cationic *trans*-diamine dichloroplatinum(II) complexes with piperazine ligands have been prepared that exhibit significant cytotoxic activity against cisplatin resistant ovarian cancer cells.[23] Moreover, these platinum-piperazine complexes are taken up by cancer cells much more rapidly and bind to cellular DNA and to calf thymus DNA much faster than cisplatin.

During the course of preparation of structurally modified PBDs, it has been observed that in the literature no effort has been made to prepare and investigate PBD dimers linked through a piperazine spacer. It was considered worthwhile to prepare and evaluate PBD dimers linked through a piperazine moiety side-armed with symmetrical alkane spacers at the C8-position that contain two DC-81 subunits. Moreover, the main objective to incorporate a piperazine moiety is to improve the bioavailability and DNA binding ability of such PBD dimers. Therefore, we herein report the synthesis and biological evaluation of novel PBD dimers linked through a piperazine moiety.

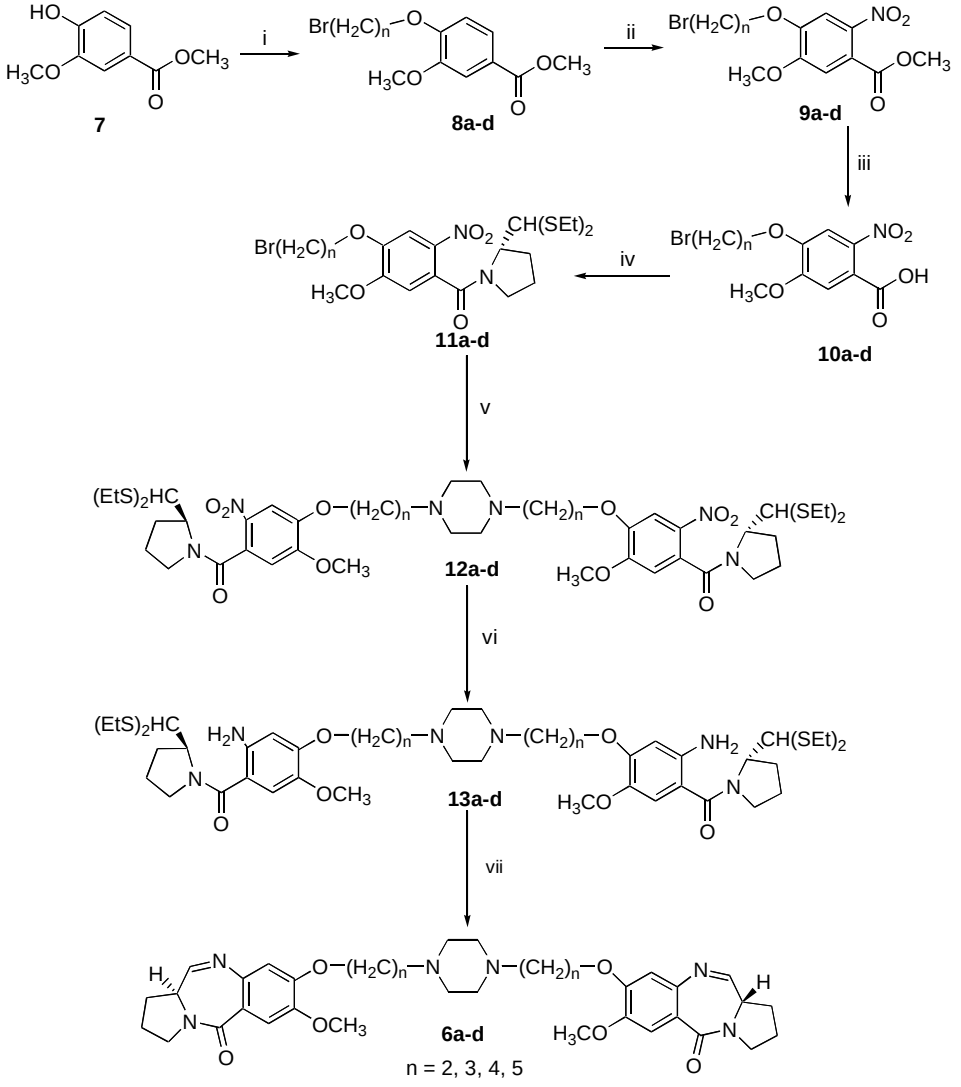
2. Results and discussion

2.1. Chemistry

Synthesis of PBD dimers linked through piperazine moiety has been carried out by employing the commercially available vanillin. Oxidation of vanillin followed by esterification employing literature procedure[24] provides the starting material vanillin methyl ester (**7**). The monoalkylation of **7** has been achieved by using three molar equivalents of the dibromoalkanes. Nitration of **8a**–**d** followed by ester hydrolysis and coupling of (2*S*)-pyrrolidine-carboxaldehyde diethyl thioacetal[25] affords **11a**–**d**. Dimerization of **11a**–**d** has been carried out by employing piperazine in the presence of potassium carbonate in refluxing acetone to provide the dimers (**12a**–**d**) linked through a piperazine moiety. These nitro dimers have been reduced with tin chloride to give the amino dimers (**13a**–**d**). Finally, deprotection of the

thioacetal group employing the literature procedure[12,26] affords the desired target molecules **6a–d** (Scheme 1).

2.1.1. Thermal denaturation studies. The DNA binding ability of the novel piperazine-linked PBD dimers (**6a–d**) was investigated by thermal denaturation studies using calf thymus (CT) DNA. The studies for these compounds (**6a–d**) were carried out by DNA/ligand molar ratios of 5:1. The increase in the helix melting temperature (ΔT_m) for each compound was examined after 0, 18, and 36 h incubation at 37 °C. Data for compounds **1** and **4** are included in Table 1 for comparison. The compound **6a** elevates the helix melting temperature of the CT-DNA by 24.0 °C after incubation at 37 °C for 18 h. The naturally occurring DC-81 (**1**) gives a ΔT_m of 0.7 °C, whereas synthetic DC-81 dimer **4** (DSB-120) gives a ΔT_m of 15.1 °C under identical experimental conditions. This result illustrates the significant effect of introducing the piperazine moiety side-armed with symmetrical alkane spacers between the two DC-81 PBD subunits. Further, it is interesting to observe that as the linker chain increases from two to four carbons there is a decrease in the DNA binding ability, while on further increase to five carbon chain the DNA stabilization is moderately enhanced, as in the case of **6d**. These results indicate that incorporation of a piperazine moiety in the alkane spacer linker enhances the DNA binding potential significantly particularly in the case of **6a** (Table 1). Melting temperature (ΔT_m) curves of CT-DNA alone and for a representative ligand complex with CT-DNA for compound **6a** have been illustrated in Figure 2. In comparison to DC-81 and the previously synthesized PBD dimer DSB-120, these PBD dimers linked through a piperazine moiety in its alkane chain spacer are likely to possess better hydrophobic interactions with the DNA to make them more stable ligands. In this process, the linker length could play an important role in the increase or decrease in the binding ability based on the favorability of such interactions. Furthermore,



Scheme 1. Reagents and conditions: (i) $Br(CH_2)_nBr$, K_2CO_3, CH_3COCH_3, reflux, 48 h. (ii) $SnCl_4/HNO_3$, CH_2Cl_2, −25 °C, 5 min. (iii) 2 N LiOH, THF, MeOH, H_2O (3:1:1), rt, 12 h. (iv) $SOCl_2$ then DMF, THF, H_2O, 2(*S*)-pyrrolidine-carboxaldehyde diethylthioacetal, Et_3N, 3 h. (v) Piperazine, K_2CO_3, CH_3COCH_3, reflux, 48 h. (vi) $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 1.5 h. (vii) $Bi(OTf)_3{\cdot}xH_2O$ or $HgCl_2$, $CaCO_3$, CH_3CN–H_2O (4:1), 3–8 h.

Table 1. Thermal denaturation data for piperazine-linked PBD dimers with calf thymus (CT) DNA

PBD dimers	[PBD]:[DNA] molar ratio[b]	ΔT_m[a] (°C) after incubation at 37 °C for		
		0 h	18 h	36 h
6a	1:5	21.8	24.0	25.0
6b	1:5	14.6	16.1	16.2
6c	1:5	10.4	14.5	15.4
6d	1:5	18.4	18.4	18.4
DSB-120 (**4**)	1:5	10.2	15.1	15.4
DC-81 (**1**)	1:5	0.3	0.7	0.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.6 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ± 0.1–0.2 °C.
[b] For a 1:5 molar ratio of [PBD]/[DNA], where CT-DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01].

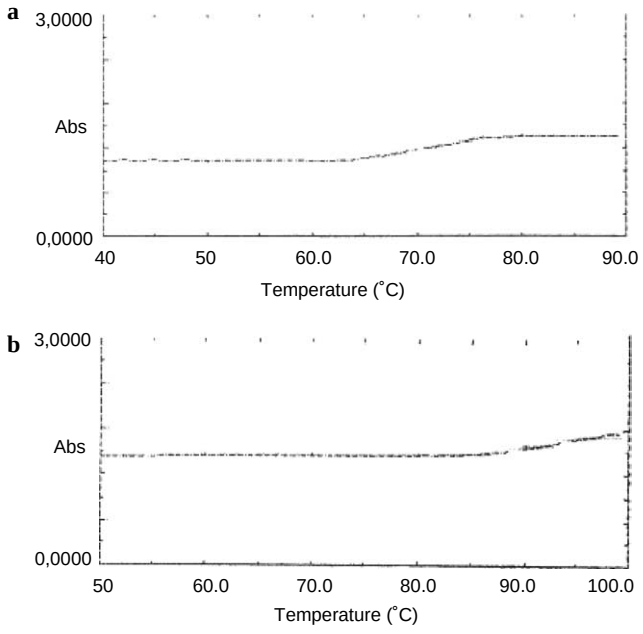


Figure 2. Thermal melting curves for CT-DNA and its ligand complex [CT-DNA without ligand (a), CT-DNA + compound **6a** (b)].

Table 1 shows that the T_m for global melting of this duplex DNA increases upon incubation in most of these dimers. This is expected due to initial non-covalent recognition and binding of the molecule to its favored DNA sites and then followed by slower covalent reversible fixation of the bound ligand.

2.1.2. Cytotoxicity. These compounds **6a**–**d** were evaluated for in vitro activity against sixty human tumor cell lines, derived from nine cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancers). The concentration causing 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI, 0% growth), and 50% cell death (LC_{50}, −50% growth) compared with the control was calculated (Table 2). The mean graph midpoint values of $\log_{10}$ TGI, and $\log_{10}$ LC_{50} as well as $\log_{10}$ GI_{50} for **6a**–**d** are listed in Table 3. However, the cytotoxicity (IC_{50}) of compounds **1** and **4** has been reported as 0.33 and 0.0005 μM. In general, all the compounds are active but compounds **6b** and **6c** show significant cytotoxic activity against some human cancer cell lines.

It is observed from the data given in Table 3 that the length of the linker plays an important role for the cytotoxic activity. Interestingly, as the alkane chain increases from two to four, the cytotoxic activity markedly increases. However, the increase of the carbon spacer to further longer chain (five carbon) in the case of **6d** moderately reduces the cytotoxicity nevertheless comparable to **6b**. The cytotoxicity data cannot be correlated to the DNA thermal denaturation values. As demonstrated by the mean graph pattern for compound **6c** (Fig. 3), it is seen that it exhibits not only significant activity but more sen-

Table 3. $\log_{10}$ GI_{50} $\log_{10}$ TGI and $\log_{10}$ LC_{50} mean graph midpoints (MG_MID) of in vitro cytotoxicity data for the compounds **6a**–**d** against human tumor cell lines

Compound	$\log_{10}$ GI_{50}	$\log_{10}$ TGI	$\log_{10}$ LC_{50}
6a	−4.69	−4.16	−4.03
6b	−5.19	−4.22	−4.01
6c	−7.70	−5.95	−4.47
6d	−5.14	−4.26	−4.04

Table 2. Log GI_{50} (concentration in mol/L causing 50% growth inhibition) values for piperazine-linked PBD dimers[a]

Cancer	Compound **6a**	Compound **6b**	Compound **6c**	Compound **6d**
Leukemia	−5.70	−6.69	−7.81	−5.48
Non-small-cell lung	−4.57	−6.60	−7.68	−5.47
Colon	−4.58	−5.78	−7.66	−5.59
CNS	−4.80	−5.71	−7.97	−5.45
Melanoma	−4.70	−5.47	−7.78	−5.62
Ovarian	−4.35	−5.36	−7.54	−5.32
Renal	−4.60	−5.70	−7.59	−6.88
Prostate	−4.40	−5.57	−7.31	−5.39
Breast	−4.53	−6.07	−7.58	−5.49

[a] Each cancer type represents the average of six to eight different cancer cell lines.

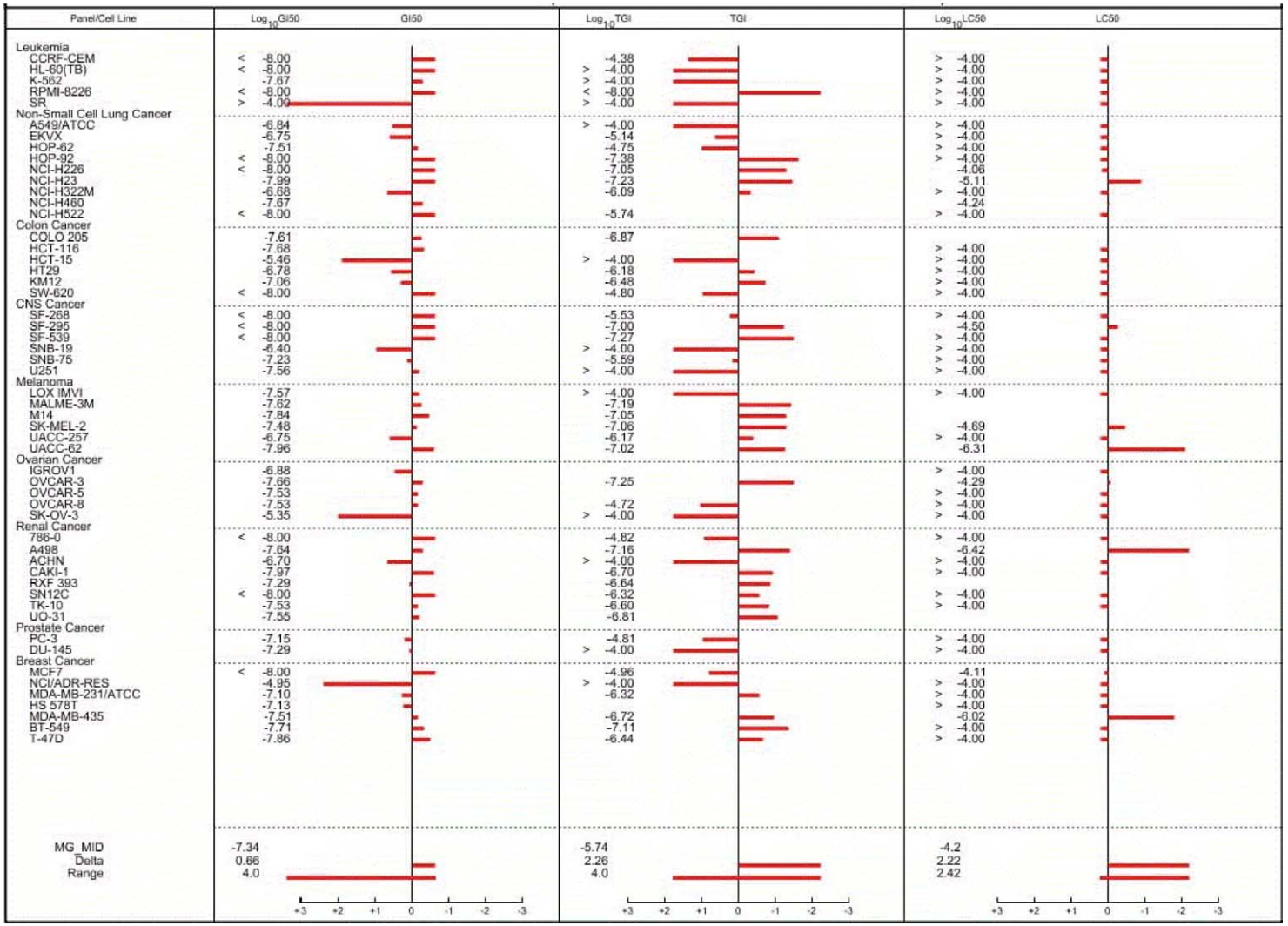


Figure 3. Log_{10} GI_{50}, Log_{10} TGI, and Log_{10} LC_{50} data from the NCI 60 cell line screen for PBD dimer **6c**.

sitivity for certain cancer cell lines and has been selected for in vivo evaluation.

3. Conclusion

Introduction of a piperazine moiety in the alkane spacer of the PBD dimers increased the DNA binding activity considerably in one of the PBD dimers **6a**. More importantly, these newly designed piperazine incorporated PBD dimers possess interesting and promising in vitro growth inhibition in the 60 cancer cell line assay. Therefore, inclusion of a piperazine moiety in the linker spacer not only enhances the potency of such PBD dimers but also their solubility profile. The in vitro cytotoxicity results obtained from this investigation will also be helpful in the design of improved interstrand DNA cross-linking molecules.

4. Experimental

4.1. Synthetic chemistry

Reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 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), acetone (potassium permanganate), and acetonitrile (phosphorous pentoxide).

^{1}H NMR spectra were recorded on a Varian Gemini 200 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 °C, 70 eV with trap current of 200 μA, and 4 kV acceleration voltage. FABMS spectra were recorded on LSIMS-VG-AUTOSPEC-Micromass. Melting points were recorded on Electrothermal 9100 and are uncorrected.

4.2. Methyl-4-(2-bromoethoxy)-3-methoxybenzoate (8a)

To a solution of vanillin methyl ester **7** (182 mg, 1 mmol) in acetone (30 mL) were added anhydrous potassium carbonate (553 mg, 4 mmol) and 1,2-dibromoethane (561 mg, 3 mmol) and the mixture was refluxed for 48 h. The progress of the reaction was monitored by TLC. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under vacuum to get the crude product. This was further purified by column chromatography (10% EtOAc–hexane) to afford the compound **8a** as a white solid (236 mg, 82%). Mp: 110–113 °C. ^{1}H NMR ($CDCl_3$): δ 3.64 (t, 2H, J = 6.2 Hz), 3.90 (s, 3H), 3.95 (s, 3H), 4.19 (t, 2H, J = 6.3 Hz), 6.88 (d, 1H, J = 6.1 Hz), 7.50 (s, 1H), 7.65 (d, 1H, J = 8.4 Hz). MS (EI) m/z 289 $[M]^{+\cdot}$.

4.3. Methyl-4-(3-bromopropoxy)-3-methoxybenzoate (8b)

The compound **8b** was prepared following the method described for the compound **8a**, employing vanillin methyl ester **7** (182 mg, 1 mmol) and 1,3-dibromopropane (605 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **8b** as a white solid (260 mg, 86%). Mp: 117–118 °C. ^{1}H NMR ($CDCl_3$): δ 2.37–2.42 (m, 2H), 3.62 (t, 2H, J = 6.2 Hz), 3.92 (s, 3H), 3.96 (s, 3H), 4.20 (t, 2H, J = 6.1 Hz), 6.85 (d, 1H, J = 8.3 Hz), 7.50 (s, 1 H), 7.65 (d, 1H, J = 8.5 Hz). MS (EI) m/z 304 $[M+1]^{+\cdot}$.

4.4. Methyl-4-(4-bromobutoxy)-3-methoxybenzoate (8c)

The compound **8c** was prepared following the method described for the compound **8a**, employing vanillin methyl ester **7** (182 mg, 1 mmol) and 1,4-dibromobutane (648 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **8c** as a white solid (260 mg, 82%). Mp: 122–124 °C. ^{1}H NMR ($CDCl_3$): δ 1.98–2.20 (m, 4H), 3.50 (t, 2H, J = 6.4 Hz), 3.93 (s, 3H), 3.96 (s, 3H), 4.15 (t, 2H, J = 6.3 Hz), 6.82 (d, 1H, J = 8.2 Hz), 7.50 (s, 1H), 7.60 (d, 1H, J = 8.4 Hz). MS (EI) m/z 317 $[M]^{+\cdot}$.

4.5. Methyl-4-(5-bromopentyloxy)-3-methoxybenzoate (8d)

The compound **8d** was prepared following the method described for the compound **8a**, employing vanillin methyl ester **7** (182 mg, 1 mmol) and 1,5-dibromopentane (689 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **8d** as a white solid (275 mg, 83%). Mp: 125–127 °C. ^{1}H NMR ($CDCl_3$): δ 1.62–2.05 (m, 6H), 3.45 (t, 2H,J = 6.4 Hz), 3.93 (s, 3H), 3.96 (s, 3H), 4.15 (t, 2H,J = 6.3 Hz), 6.82 (d, 1H, J = 8.2 Hz), 7.55 (s, 1H), 7.60 (d, 1H, J = 8.8 Hz). MS (EI) m/z 331 $[M]^{+\cdot}$.

4.6. Methyl-4-(2-bromoethoxy)-5-methoxy-2-nitrobenzoate (9a)

A freshly prepared mixture of stannic chloride (260 mg, 1.156 mmol) and fuming nitric acid (98 mg, 1.56 mmol) in dichloromethane was added dropwise for over 5 min with stirring to a solution of methyl-4-(2-bromoethoxy)-3-methoxybenzoate (**8a**) (289 mg, 1 mmol) in dichloromethane (30 mL) at −25 °C (dry ice/carbon tetrachloride). The mixture was maintained at −25 °C for a further 5 min, quenched with water (20 mL), and then allowed to return to room temperature. The organic layer was separated and the aqueous layer was extracted with dichloromethane (2 × 20 mL). The combined organic phase was dried over anhydrous Na_2SO_4, evaporated under vacuum, and purified by column chroma-

tography (20% EtOAc–hexane) to afford the compound **9a** as an oily liquid (300 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 3.65 (t, 2H, J = 6.2 Hz), 3.90 (s, 3H), 3.95 (s, 3H), 4.19 (t, 2H, J = 6.3 Hz), 7.05 (s, 1H), 7.42 (s, 1H). MS (EI) m/z 334 $[M]^{+\cdot}$.

4.7. Methyl-4-(3-bromopropoxy)-5-methoxy-2-nitrobenzoate (9b)

Compound **8b** (303 mg, 1 mmol) was nitrated by the similar method described earlier for **9a** to afford the compound **9b** as a pale yellow oily liquid (313 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 2.30–2.45 (m, 2H), 3.62 (t, 2H, J = 6.3 Hz), 3.89 (s, 3H), 3.96 (s, 3H), 4.12 (t, 2H, J = 6.1 Hz), 7.10 (s, 1H), 7.22 (s, 1H). MS (EI) m/z 347 $[M-1]^{+\cdot}$.

4.8. Methyl-4-(4-bromobutoxy)-5-methoxy-2-nitrobenzoate (9c)

Compound **8c** (317 mg, 1 mmol) was nitrated by the similar method described earlier for **9a** to afford the compound **9c** as a pale yellow oily liquid (218 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 1.95–2.10 (m, 4H), 3.45 (t, 2H, J = 6.2 Hz), 3.88 (s, 3H), 3.95 (s, 3H), 4.10 (t, 2H, J = 6.10 Hz), 7.05 (s, 1H), 7.40 (s, 1H). MS (EI) m/z 362 $[M]^{+\cdot}$.

4.9. Methyl-4-(5-bromopentyloxy)-5-methoxy-2-nitrobenzoate (9d)

Compound **8d** (331 mg, 1 mmol) was nitrated by the similar method described earlier for **9a** to afford the methyl 4-(5-bromopentyloxy)-5-methoxy-2-nitrobenzoate **9d** as a light yellow oily liquid (345 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 1.60–2.00 (m, 6H), 3.40 (t, 2H), 3.90 (s, 3H), 3.95 (s, 3H), 4.20 (t, 2H), 7.05 (s, 1H), 7.40 (s, 1H). MS (EI) m/z 376 $[M]^{+\cdot}$.

4.10. 4-(2-Bromoethoxy)-5-methoxy-2-nitrobenzoic acid (10a)

A solution of 2 N lithium hydroxide monohydrate (1.221 mL) was added to a solution of methyl 4-(2-bromoethoxy)-5-methoxy-2-nitrobenzoate (**9a**) (334 mg, 1 mmol) in THF–H_2O–MeOH (3:1:1) and the mixture was stirred at room temperature for 12 h. After most of the THF and methanol had been evaporated, the aqueous phase was acidified with 12 N HCl to pH 7 and extracted with ethyl acetate to give the compound **10a** as a white solid (288 mg, 90%). Mp 120–125 °C. ^{1}H NMR ($CDCl_3$): δ 3.64 (t, 2H, J = 6.3 Hz), 3.98 (s, 3H), 4.20 (t, 2H, J = 6.2 Hz), 7.20 (s, 1H), 7.42 (s, 1H). MS (EI) m/z 320 $[M]^{+\cdot}$.

4.11. 4-(3-Bromopropoxy)-5-methoxy-2-nitrobenzoic acid (10b)

The compound **10b** was prepared by the same method described earlier for **10a** employing **9b** (348 mg, 1 mmol), which gave **10b** as a white solid (297 mg, 89%). Mp 118–120 °C. ^{1}H NMR ($CDCl_3$): δ 2.28–2.48 (m, 2H), 3.6 (t, 2H, J = 6.4 Hz), 3.98 (s, 3H), 4.25 (t, 2H, J = 6.2 Hz), 7.20 (s, 1H), 7.40 (s, 1H). MS (EI) m/z 334 $[M]^{+\cdot}$.

4.12. 4-(4-Bromobutoxy)-5-methoxy-2-nitrobenzoic acid (10c)

The compound **10c** was prepared by the same method described earlier for **10a** employing **9c** (362 mg, 1 mmol), which gave **10c** as a white solid (324 mg, 93%). Mp 176–178 °C. ^{1}H NMR ($CDCl_3$): δ 2.0–2.15 (m, 4H), 3.50 (t, 2H, J = 6.3 Hz), 3.96 (s, 3H), 4.15 (t, 2H, J = 6.1 Hz), 7.18 (s, 1H), 7.38 (s, 1H). MS (EI) m/z 348 $[M]^{+\cdot}$.

4.13. 4-(5-Bromopentyloxy)-5-methoxy-2-nitrobenzoic acid (10d)

The compound **10d** was prepared by the same method described earlier for **10a** employing **9d** (376 mg, 1 mmol), which gave **10d** as a white solid (330 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 1.9–2.1 (m, 6H), 3.45 (t, 2H, J = 6.4 Hz), 3.95 (s, 3H), 4.1–4.15 (t, 2H, J = 6.2 Hz), 7.18 (s, 1H), 7.38 (s, 1H) MS (EI) m/z 362 $[M]^{+\cdot}$.

4.14. (2*S*)-*N*-[4-(2-Bromoethoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (11a)

DMF was added to a stirred suspension of compound **10a** (320 mg, 1 mmol) and thionyl chloride (476 mg, 4 mmol) in dry benzene (15 mL) and the stirring was continued for 6 h. The benzene was evaporated under vacuum and the resultant oil was dissolved in dry THF (20 mL) and added dropwise over a period of 30 min to a stirred suspension of (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (205 mg, 1 mmol), triethylamine (303 mg, 3 mmol), and ice water (2 mL) in dry THF cooled in an ice bath. After the addition was completed, the reaction mixture was brought to ambient temperature and stirred for an additional hour. The THF was evaporated under vacuum and the aqueous layer was washed with ethyl acetate (10 mL). The aqueous phase was then adjusted to pH 3 using 6 N HCl and extracted with ethyl acetate and washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The crude product was purified by column chromatography (30% EtOAc–hexane) to afford the compound **11a** as an oily liquid (456 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 1.22–1.40 (m, 6H), 1.82–2.50 (m, 4H), 2.60–2.90 (m, 4H), 3.19–3.28 (m, 2H), 3.65 (t, 2H, J = 6.2 Hz), 3.95 (s, 3H), 4.35 (t, 2H, J = 6.3 Hz), 4.59–4.71 (m, 1H), 4.82 (d, 1H, J = 4.2 Hz), 6.78 (s, 1H), 7.70 (s, 1H). MS (FAB) 507 $[M]^{+\cdot}$.

4.15. (2*S*)-*N*-[4-(3-Bromopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (11b)

The compound **11b** was prepared following the method described for the compound **11a** by employing the compound **10b** (334 mg, 1 mmol) and (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal, which gave the product as an oily liquid (464 mg, 89%). ^{1}H NMR ($CDCl_3$): δ

1.30–1.42 (m, 6H), 1.85–2.50 (m, 6H), 2.70–2.85 (m, 4H), 3.20–3.35 (m, 2H), 3.65 (t, 2H, $J = 6.20$ Hz), 3.95 (s, 3H), 4.29 (t, 2H, $J = 6.3$ Hz), 4.63-4.75 (m, 1H), 4.85 (d, 1H, $J = 4.2$ Hz), 6.79 (s, 1H), 7.70 (s, 1H). MS (FAB) 521 $[M]^{+\cdot}$.

4.16. (2*S*)-*N*-[4-(4-Bromobutoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (11c)

The compound **11c** was prepared following the method described for the compound **11a** by employing the compound **10c** (348 mg, 1 mmol) and (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal, which gave **11c** as an oily liquid (476 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 1.30–1.43 (m, 6H), 1.85–2.38 (m, 8H), 2.69–2.88 (m, 4H), 3.20–3.32 (m, 2H), 3.51 (t, 2H, $J = 6.4$ Hz), 3.97 (s, 3H), 4.16 (t, 2H, $J = 6.4$ Hz), 4.63–4.76 (m, 1H), 4.88 (d, 1H, $J = 6.4$ Hz), 6.79 (s, 1H), 7.67 (s, 1H). MS (FAB) 535 $[M]^{+\cdot}$.

4.17. (2*S*)-*N*-[4-(5-Bromopentyloxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (11d)

The compound **11d** was prepared following the method described for the compound **11a** by employing the compound **10d** (362 mg, 1 mmol) and (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal, which gave **11d** as an oily liquid (505 mg, 92%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.43 (m, 6H), 1.60–2.30 (m, 10H), 2.63–2.82 (m, 4H), 3.18–3.30 (m, 2H), 3.42 (t, 2H, $J = 6.20$ Hz), 3.96 (s, 3H), 4.10 (t, 2H, $J = 6.10$ Hz), 4.60–4.70 (m, 1H), 4.85 (d, 1H, $J = 4.20$ Hz), 6.78 (s, 1H), 7.62 (s, 1H). MS (FAB) 549 $[M]^{+\cdot}$.

4.18. 1,1′-[[1,4-Di(ethane-1,2-diyl)hexahydropiperazine]dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (12a)

To a solution of (2*S*)-*N*-[4-(2-bromoethoxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal (**11a**) (507 mg, 1 mmol) in dry acetone (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the piperazine (43 mg, 0.5 mmol). The reaction mixture was refluxed for 48 h. The reaction was monitored by TLC using ethyl acetate/hexane (8:2) as a solvent system. The potassium carbonate was removed by suction filtration and the solvent was evaporated under vacuum. The crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12a** as a white solid (845 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 1.29–1.41 (m, 12H), 1.70–2.39 (m, 8H), 2.61–2.95 (m, 20H), 3.19–3.30 (m, 4H), 3.92 (s, 6H), 4.20 (t, 4H, $J = 6.2$ Hz), 4.61–4.72 (m, 2H), 4.82 (d, 2H, $J = 4.30$ Hz), 6.78 (s, 2H), 7.72 (s, 2H). MS (FAB) 939 $[M]^{+\cdot}$.

4.19. 1,1′-[[1,4-Di(propane-1,3-diyl)hexahydropiperazine]dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (12b)

The compound **12b** was prepared following the method described for the compound **12a**, employing piperazine and **11b** (521 mg, 1 mmol), and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12b** as a white solid (860 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.28–1.41 (m, 12H), 1.90–2.32 (m, 12H), 2.42–2.88 (m, 20H), 3.18–3.32 (m, 4H), 3.96 (s, 6H), 4.18 (t, 4H, $J = 6.4$ Hz), 4.62–4.75 (m, 2H), 4.82 (d, 2H, $J = 4.30$ Hz), 6.75 (s, 2H), 7.67 (s, 2H). MS (FAB) 967 $[M+1]^{+\cdot}$.

4.20. 1,1′-[[1,4-Di(butane-1,4-diyl)hexahydropiperazine]dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (12c)

The compound **12c** was prepared according to the method described for the compound **12a**, employing piperazine and **11c** (535 mg, 1 mmol), and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12c** as a white solid (875 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 1.30–1.43 (m, 12H), 1.70–2.38 (m, 16H), 2.51–2.66 (m, 20H), 3.20–3.32 (m, 4H), 3.97 (s, 6H), 4.18 (t, 4H, $J = 6.2$ Hz), 4.62–4.76 (m, 2H), 4.87 (d, 2H, $J = 4.20$ Hz), 6.79 (s, 2H), 7.63 (s, 2H). MS (FAB) 995 $[M]^{+\cdot}$.

4.21. 1,1′-[[1,4-Di(pentane-1,5-diyl)hexahydropiperazine]dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (12d)

The compound **12d** was prepared following the method described for the compound **12a**, employing piperazine and **11d** (549 mg, 1 mmol), and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12d** as a white solid (908 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 12H), 1.50–2.35 (m, 20H), 2.45–2.82 (m, 20H), 3.15–3.30 (m, 4H), 3.96 (s, 6H), 4.06 (t, 4H, $J = 6.20$ Hz), 4.60–4.75 (m, 2H), 4.80 (d, 2H, $J = 4.20$ Hz), 6.75 (s, 2H), 7.60 (s, 2H). MS (FAB) 1023 $[M]^{+\cdot}$.

4.22. 1,1′-[[1,4-Di(ethane-1,2-diyl)hexahydropiperazine]dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (13a)

The compound **12a** (939 mg, 1 mmol) dissolved in methanol (20 mL) and $SnCl_2{\cdot}2H_2O$ (2.256 g, 10 mmol) added was refluxed for 1.5 h. The reaction mixture was cooled and the methanol was evaporated under vacuum, and the residue was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with ethyl acetate (2×30 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum to afford the crude amino diethyl thioacetal **13a** as a yellow oil (747 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 12H), 1.60–2.38 (m, 8H), 2.58–2.68 (m, 16H), 2.82 (t, 4H, $J = 6.3$ Hz), 3.57–3.70 (m, 4H), 3.75 (s, 6H), 4.09 (t, 4H, $J = 6.2$ Hz), 4.56–4.70 (m, 4H), 6.19 (s, 2H), 6.78 (s, 2H). MS (FAB) 879 $[M+H]^{+\cdot}$.

4.23. 1,1′-[[1,4-Di(propane-1,3-diyl)hexahydropiperazine]dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (13b)

The compound **13b** was prepared following the method described for the compound **13a** employing the compound **12b** (967 mg, 1 mmol) to afford the amino diethyl thioacetal **13b** as a yellow oil (802 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.19–1.40 (m, 12H), 1.90–2.35 (m, 12H), 2.45–2.88 (m, 20H), 3.56–3.65 (m, 4H), 3.95 (s, 6H), 4.00 (t, 5H, J = 6.1 Hz), 4.55–4.88 (m, 4H), 6.20 (s, 2H), 6.75 (s, 2H). MS (FAB) 907 $[M]^{+\cdot}$.

4.24. 1,1′-[[1,4-Di(butane-1,4-diyl)hexahydropiperazine]dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (13c)

The compound **13c** was prepared following the method described for the compound **13a** employing the compound **12c** (995 mg, 1 mmol) to afford the amino diethyl thioacetal **13c** as a yellow oil (794 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 12H), 1.90–2.38 (m, 16H), 2.45–2.75 (m, 20H), 3.57–3.65 (m, 4H), 3.95 (s, 6H), 4.05 (t, 4H, J = 6.2 Hz), 4.56–4.68 (m, 4H), 6.20 (s, 2H), 6.76 (s, 2H). MS (FAB) 935 $[M]^{+\cdot}$.

4.25. 1,1′-[[1,4-Di(pentane-1,5-diyl)hexahydropiperazine]dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]]-bis[pyrrolidine-2-carboxaldehyde diethyl thioacetal] (13d)

The compound **13d** was prepared following the method described for the compound **13a** employing the compound **12d** (1023 mg, 1 mmol) to afford the amino diethyl thioacetal **13d** as a yellow oil (787 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 1.20–1.40 (m, 12H), 1.90–2.38 (m, 20H), 2.45–2.82 (m, 20H), 3.57–3.65 (m, 4H), 3.96 (s, 6H), 4.05 (t, 4H, J = 6.2 Hz), 4.55–4.68 (m, 4H), 6.30 (s, 2H), 6.78 (s, 2H). MS (FAB) 963 $[M]^{+\cdot}$.

4.26. 1,1′-[[1,4-Di(ethane-1,2-diyl)hexahydropiperazine]dioxy]-bis[(11a*S*)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one] (6a)

A solution of amino thioacetal **13a** (878 mg, 1 mmol), $HgCl_2$ (1.19 g, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in acetonitrile–water (4:1) was stirred slowly at room temperature overnight until TLC indicated the complete disappearance of starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a Celite. The clear yellow organic supernatant was extracted with ethyl acetate (2×20 mL). The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (95% CH_2Cl_2–MeOH) to afford the compound **6a** as a pale yellow solid (410 mg, 65%). This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.$[\alpha]_D^{26} = +353.5$ (c 1.0 $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.95–2.40 (m, 8H), 2.59–2.97 (m, 12H), 3.50–3.87 (m, 6H), 3.95 (s, 6H), 4.15–4.28 (m, 4H), 6.78 (s, 2H), 7.50 (s, 2H), 7.66 (d, 2H, J = 4.4 Hz). ^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 22.4, 24.0, 29.2, 39.0, 39.3, 39.6, 39.9, 40.1, 40.4, 40.7, 46.8, 53.5, 56.8, 66.8, 110.7, 111.7, 120.2, 140.9, 147.3, 150.5, 163.7. MS (FAB) 631 $[M+1]^{+\cdot}$. Anal. Calcd for $C_{34}H_{42}N_6O_6$: C, 64.75; H, 6.71; N, 13.32. Found: C, 64.69; H, 6.53; N, 13.22.

4.27. 1,1′-[[1,4-Di(propane-1,3-diyl)hexahydropiperazine]dioxy]-bis[(11a*S*)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one] (6b)

The compound **6b** was prepared following the method described for the compound **6a** employing **13b** (907 mg, 1 mmol), to afford the compound **6b** as a pale yellow solid (415 mg, 63%). $[\alpha]_D^{25}$ +329.0 (c 1.01 $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.61–2.18 (m, 12H), 2.23–2.75 (m, 12H), 3.40–3.70 (m, 6H), 3.92 (s, 6H), 4.00–4.21 (m, 4H), 6.75 (s, 2H), 7.48 (s, 2H), 7.60 (d, 2H, J = 4.4 Hz). MS (FAB) 659 $[M+1]^{+\cdot}$. Anal. Calcd for $C_{36}H_{46}N_6O_6$: C, 65.71; H, 7.05; N, 12.77. Found: C, 65.67; H, 6.98; N, 12.70.

4.28. 1,1′-[[1,4-Di(butane-1,4-diyl)hexahydropiperazine]dioxy]-bis[(11a*S*)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one] (6c)

The compound **6c** was prepared according to the method described for the compound **6a** employing **13c** (935 mg, 1 mmol), to afford the compound **6c** as a pale yellow solid (419 mg, 61%). $[\alpha]_D^{26}$ +315.05 (c 1.0 $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.61–2.18 (m, 8H), 2.23–2.75 (m, 20H), 3.40–3.70 (m, 6H), 3.95 (s, 6H), 4.10–4.23 (m, 4H), 6.75 (s, 2H), 7.48 (s, 2H), 7.60 (d, 1H, J = 4.3 Hz). MS (FAB) 687 $[M+1]^{+\cdot}$. Anal. Calcd for $C_{38}H_{50}N_6O_6$: C, 66.53; H, 7.34; N, 12.25. Found: C, 66.45; H, 7.34; N, 12.17.

4.29. 1,1′-[[1,4-Di(pentane-1,5-diyl)hexahydropiperazine]dioxy]bis[(11a*S*)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one] (6d)

The compound **6d** was prepared following the method described for the compound **6a** employing **13d** (963 mg, 1 mmol), to afford the compound **6d** as a pale yellow solid (450 mg, 63%). $[\alpha]_D^{26}$ +343.5 (c 1.0 $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.55–2.38 (m, 20H), 2.60–3.15 (m, 12H), 3.45–3.80 (m, 6H), 3.96 (s, 6H), 4.01–4.20 (m, 4H), 6.81 (s, 2H), 7.50 (s, 2H), 7.78 (d, 1H, J = 4.6 Hz). ^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 22.7, 23.4, 27.6, 28.8, 38.5, 38.8, 39.1, 39.3, 39.6, 39.9, 40.2, 45.9, 50.3, 53.0, 56.5, 109.8, 110.8, 115.0, 119.5, 139.8, 146.9, 149.9, 163.7. MS (FAB) 715 $[M+1]^{+\cdot}$. Anal. Calcd for $C_{40}H_{54}N_6O_6$: C, 67.20; H, 7.61; N, 11.76. Found: C, 67.21; H, 7.58; N, 11.71.

4.30. Thermal denaturation studies

The DNA binding affinity of the novel piperazine linked PBD dimers (**6a**–**d**) has been evaluated through thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using modified reported proce-

dure.[27,28] Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM Na_2EDTA, pH 7.00 ± 0.01) counting 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 are incubated at 37 °C for 0, 18, and 36 h prior to analysis. Samples are monitored at 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller and were heated at 1 °C /min in the range of 40–95 °C. DNA helix coil transition temperatures (T_m) were obtained from the maxima in the $d(A_{260})/dT$ derivative plots. Drug-induced alterations in DNA melting temperatures 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 69.6 ± 0.01.

4.31. In vitro evaluation of cytotoxic activity

The compounds **6a**–**d** were evaluated for in vitro activity against sixty human tumor cell lines, derived from nine cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancers). For each compound, dose–response curves against each cell line were measured at a minimum of five concentrations at 10-fold dilutions. A protocol of 48 h continuous drug exposure was used, and a sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth.

Acknowledgments

We thank National Cancer Institute, Maryland, for in vitro anticancer assay in human cancer cell lines. P.S.M.M.R. and D.R.S.R. are grateful to CSIR, New Delhi, for the award of Research Fellowship.

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