Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 3895–3906
Bioorganic & Medicinal Chemistry

Phosphonate-linked pyrrolo[2,1-*c*][1,4]benzodiazepine conjugates: Synthesis, DNA-binding affinity and cytotoxicity

Ahmed Kamal,[a,*] P. Praveen Kumar,[a] B. N. Seshadri,[a] O. Srinivas,[a] M. Shiva Kumar,[a] Subrata Sen,[b] Nisha Kurian,[b] Aarti S. Juvekar[b] and Surekha M. Zingde[b]

[a]*Biotransformation Laboratory, Division of Organic Chemistry, Indian Institute of Chemical Technology, Hyderabad, Andhra Pradesh 500 007, India*
[b]*Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Khargar, Navi Mumbai 410 208, India*

Received 23 November 2007; revised 22 January 2008; accepted 23 January 2008
Available online 30 January 2008

Abstract—Pyrrolobenzodiazepine-diethylphosphonate conjugates have been designed and synthesized that link through two different types of spacers that are simple alkane chain and also a piperazine moiety side-armed with the alkane chains. These pyrrolobenzodiazepine conjugates have exhibited remarkable DNA-binding affinity and improved solubility in water, a representative compound **7d** showing promising in vitro cytotoxicity.
© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

In recent years, combination chemotherapy with different mechanisms of action is one of the methods that are 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. Organophosphorous compounds are important substrates in the study of biochemical processes,[1] and the well-known class of phosphoro-organic alkylating drugs (cyclophosphamide[2] and thiotepa[3]) utilized in cancer chemotherapy in the last decade. α-Aminophosphonates and their derivatives (**1**) are important compounds possessing diverse and useful biological activities ranging from agriculture to medicine (anticancer agents,[4] antibiotics,[5] and enzyme inhibitors[6]). These are bioisosteres of natural amino acids, and acting as competitive inhibitors, biological properties are mostly associated with the tetrahedral structure of the phosphonyl group acting as a transition-state analogue. In addition, the alkylating properties of phosphonic esters have been exhaustively described in many studies[7,8] and have been a subject of many patents.[9] Moreover, phosphonic esters are either synthetic or isolated from *Streptomyces* species.[10] On the other hand, phosphorus-containing hybrids such as coumarin-phosphonate[11] and chromone-phosphonate[12] hybrids are novel group of compounds possessing remarkable cytotoxicity, alkylating, and anticancer activity against selected tumour cell lines. Similarly, 9-(2-phosphonylmethoxyethyl)guanine (PMEG) is an acyclic nucleoside phosphonate derivative that has demonstrated significant anticancer activity in a number of in vitro and in vivo animal model systems and is selectively active against a panel of human leukemic cells.[13] A series of new aminophosphonicacid derivatives of vinblastine (VLB) have been synthesized and evaluated for their in vitro and in vivo for antitumor activity.[14]

The imine or carbinolamine-containing pyrrlo[2,1-*c*][1,4]benzodiazepines are a family of low molecular weight antitumor antibiotics originally isolated from various *Streptomyces* species[15] and examples of which include DC-81 (**2**), anthramycin, tomaymycin and sibiromycin. These antibiotics bind selectively in the minor groove of DNA through a covalent aminal bond between the electrophilic C11 position of the PBD moiety and the nucleophilic N2-amino group of a guanine base,[16] resulting in the possible biological activity. In the literature, a large number of PBD conjugates and their dimers have been synthesized and evaluated for their biological activity, particularly for their antitumour potential.[17–19] Recently mixed imine–amide and

Keywords: Pyrrolobenzodiazepine; Diethylphosphonate; DNA-binding affinity; Cytotoxicity.
* Corresponding author. Tel.: +91 40 27193157; fax: +91 40 27193189; e-mail: ahmedkamal@iict.res.in

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.01.040

imine–amine PBD dimers have been designed and synthesized in this laboratory for investigating the contribution from the non-covalent interactions by one of the subunits in these dimers. It has been observed that the contribution by such a non-covalent component plays an important role in the overall DNA-binding affinity in such mixed-type of PBD dimers.[20,21] These findings provided further impetus to explore the combination of certain non-covalent interacting groups with a PBD moiety that led to the design and synthesis of a variety of PBD hybrids. The research in this direction in this laboratory afforded a number of such PBD hybrids with enhanced DNA-binding ability and significant anticancer potential.[22]

During the last decade, a number of piperazine derivatives have been synthesized for their chemotherapeutic use in the area of medicinal chemistry.[23] Michejda and co-workers[24] reported symmetrical bifunctional agents as a promising antitumour class of compounds with remarkable selectivity against colon cancers that possess a piperazine moiety in its linker spacer. Recently, *trans*-diamine dichloroplatinum(II) complexes with piperazine ligands have exhibited significant cytotoxicity.[25] Recently, synthesized C-8 linked *N*-methyl piperazine-PBD monomer (**3**)[26] and in its linker spacer incorporating a piperazine moiety PBD dimers (**4**)[27] has been synthesized that exhibit promising anticancer activity and increased DNA-binding ability (Fig. 1), in comparison to DC-81 and PBD dimer (DSB-120), respectively. Further, these PBD dimers when linked through a piperazine moiety in its alkane chain spacer provided better hydrophobic interactions with the DNA to make them more stable ligands. Therefore, these observations indicate that incorporation of a piperazine moiety in the alkane spacer linker enhances the DNA-binding potential significantly.[28]

Recently, we investigated the synthesis of C-8 methanesulphonate substituted pyrrolobenzodiazepines as potential antitumour agents (**5**).[29] In continuation of these efforts and the above-said aspects it is considered interesting to design and synthesize molecules based on PBD linked to phosphonate residue like diethylphosphonate with or without incorporating a piperazine moiety in the linker spacer. It is envisaged that such an investigation could unravel the intricacies underlining the role of phosphonate as well as piperazine moieties. Moreover it is well known that these moieties are very important with respect to the enhancement of DNA-binding ability as well as improvement in the bioavailability of such conjugates based on PBD ring system.

2. Results and discussion

2.1. Chemistry

The compound **6** was prepared from the (2*S*)-*N*-(4-benzyloxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethylthioacetal (**8**), which has been prepared by the literature method[28] and, this upon debenzylation gave (2*S*)-*N*-(4-hydroxy)-5-methoxy-2-nitrobenzoyl) pyr-rolidine-2-carboxaldehyde diethyl thioacetal (**9**). Etherification of hydroxy compound **9** with diethyl(3-bromopropyl)phosphonate in the presence of K_2CO_3 in acetone afforded the desired nitro precursor (2*S*) -*N*-{4-[3-(diethoxyphosphoryl)propyl]oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (**10**). Further, reduction of nitro compound **10** by using $SnCl_2$. $2H_2O$ reflux in methanol provided the amine compound **11**. The deprotection of thioacetal group with the $HgCl_2/CaCO_3$ yielded formation of the target compound **6**[30] (Scheme 1).

Similarly, synthesis of compounds **7a**–**e** has been carried out by employing hydroxy compound (**9**) as the starting material, which upon monoalkylation was achieved by employing dibromoalkanes-yielded (2*S*)-*N*-[4-(*n*-bromoalkyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetals (**12a**–**c**). These coupled with *n*-bocpiperazine provided the boc-protected compounds **13a**–**c**. Further, deprotection of boc-protected compounds **13a**–**c** employing triflouroaceticacid in dichloromethane gave corresponding amine compounds **14a**–**c**. The coupling of diethyl(*n*-bromoalkyl)phosphonates with amine compounds **14a**–**c** provided the desired other precursors (2*S*)-*N*-{4-[*n*-[4-[*n*-(diethoxyphosphoryl)-alkyl]piperazin-1-yl]alkyl]-oxy-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehyde diethyl thioacetals (**15a**–**e**). A two-step pathway achieved the final assembly of the desired target compounds **7a**–**e** in the above conventional manner (Scheme 2).

2.2. DNA interactions: thermal denaturation studies

The DNA-binding ability for these C8-linked PBD conjugates has been examined by thermal denaturation studies using calf thymus (CT) DNA. Thermal denaturation studies have shown that these compounds stabilized the thermal helix → coil or melting stabilization (ΔT_m) for the CTDNA duplex at pH 7.0, incubated for 18 h at 37 °C, where PBD/DNA molar ratio is 1:5.[31,32] Interestingly, in this study PBD conjugates have shown significant melting temperature values (4.5–10.0 °C) that are included in (Table 1). It is interesting to observe that the compounds **7a**, **7b** and **7e** have shown higher ΔT_m values, i.e., 9.0, 9.9 and 10.0 °C, respectively, where the PBD ring system and phosphonate units separated with 5 or 6 or 8 ($n + m$) carbon length of alkane chain spacers along with piperazine moiety. Another aspect which has been seen from the thermal denaturation data is that for compounds **7a** and **7e**, the ΔT_m values do not change even after incubation for 18 h. In case of compounds **7c** and **7 d** examined after 18 h of incubation the ΔT_m values are 7.1 and 7.2 °C, where those two functionalities are separated with 7 carbon length of alkane chain spacer ($n + m = 7$) along with piperazine moiety. Moreover, simple phosphonate–PBD conjugate (**6**) has shown lower DNA-binding affinity. In the same experiment *N*-methyl piperazine-PBD conjugate (**3**) and naturally occurring DC-81 (**2**) exhibited the ΔT_m values 6.9 and 0.7 °C, respectively, whereas piperazine-incorporated PBD dimers have shown ΔT_m values (15.1–20.0 °C),[27]

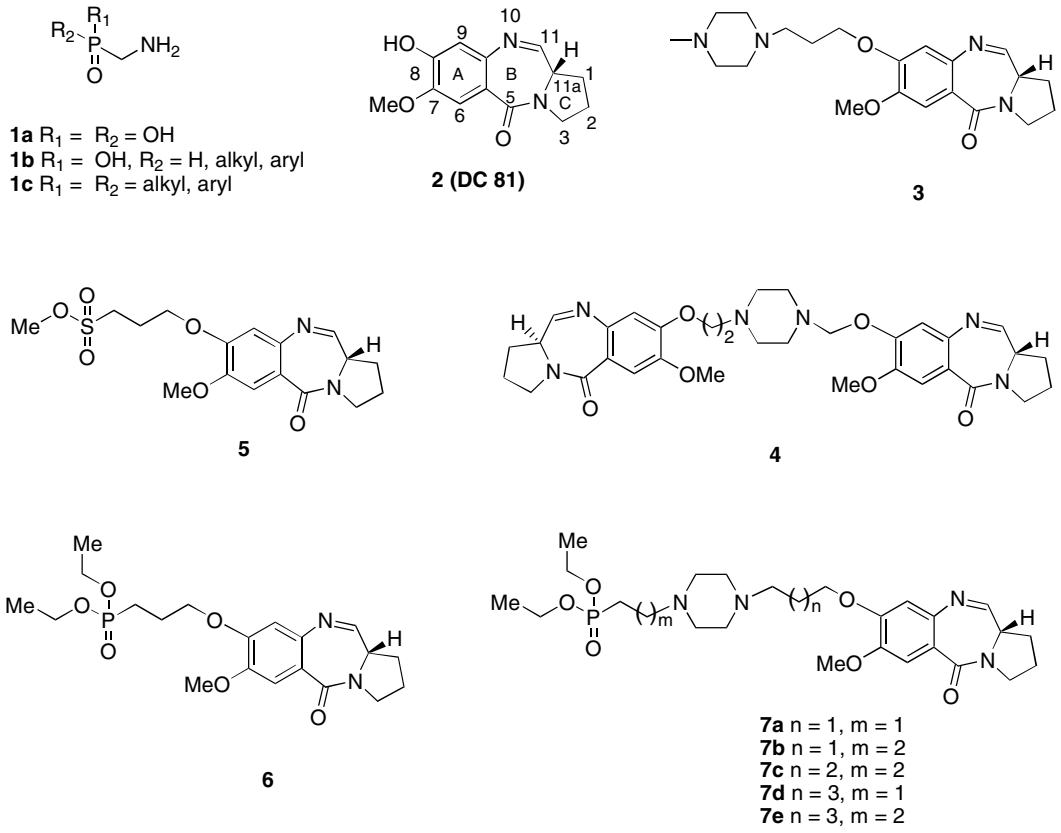
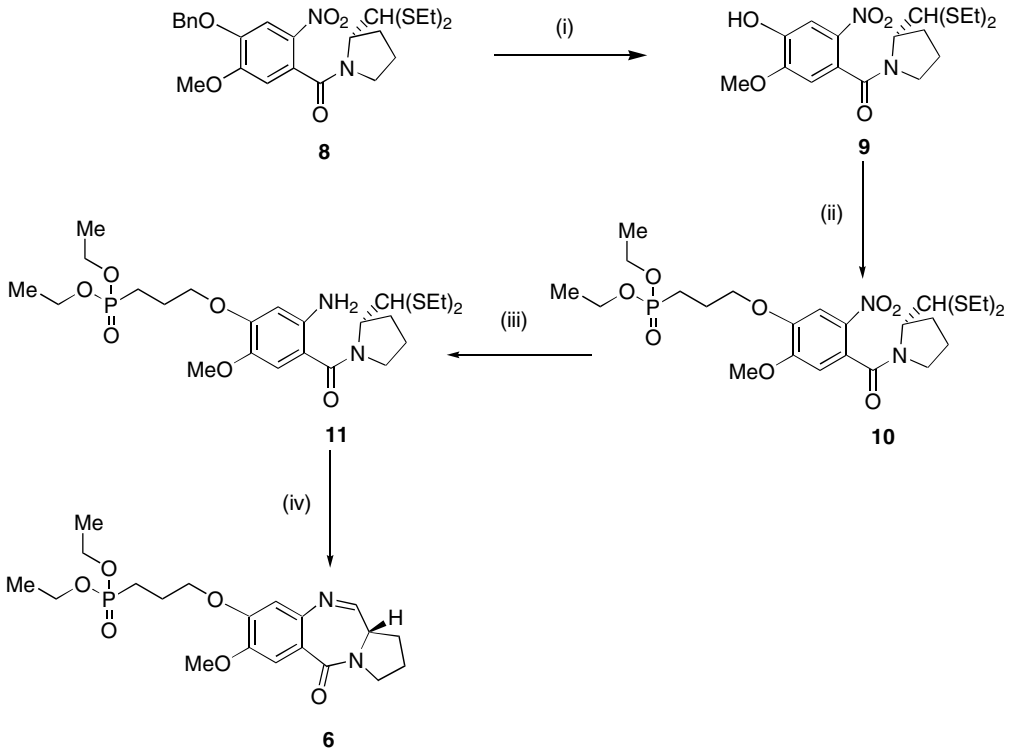
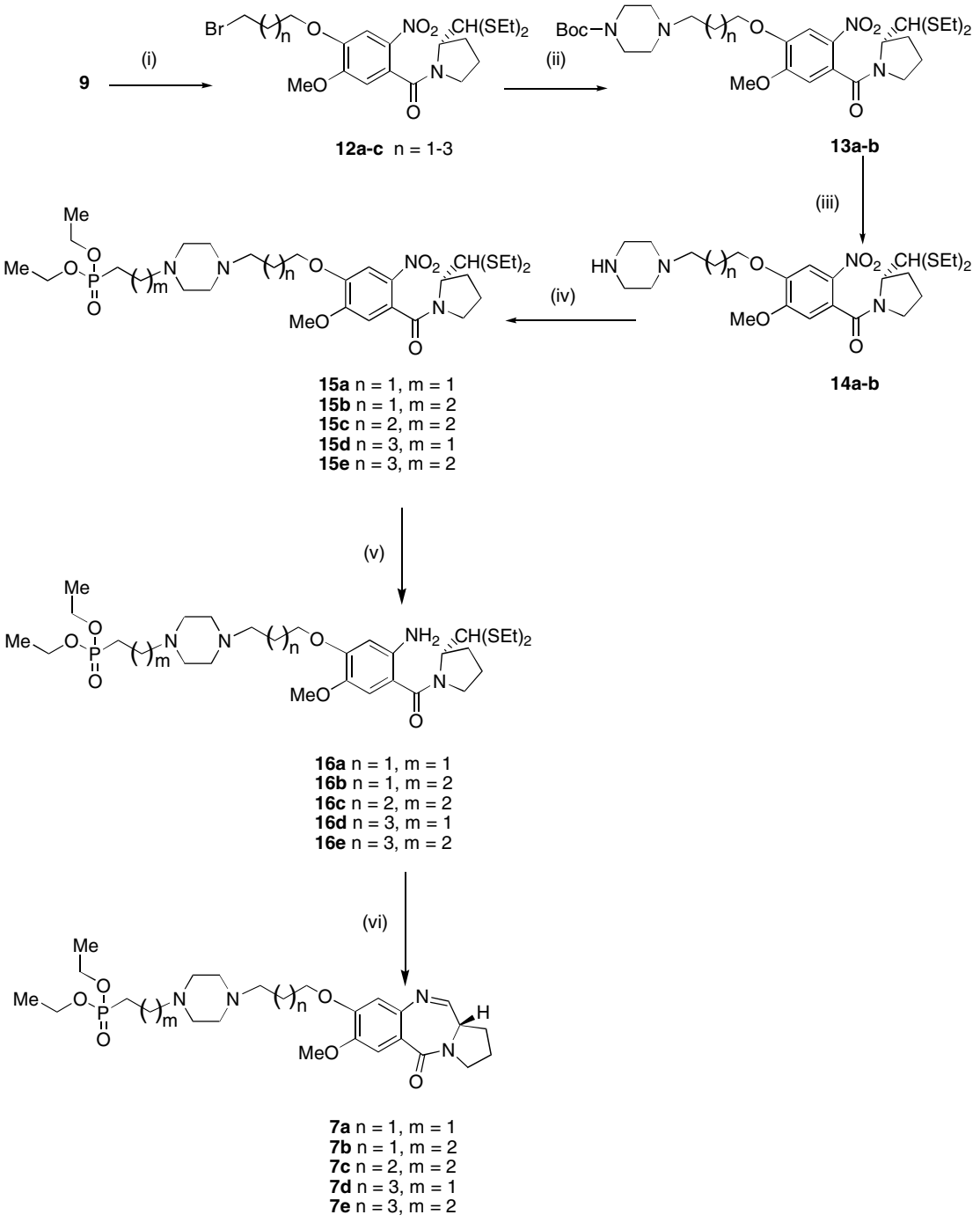


Figure 1. Chemical structures of aminophosphonic acid derivatives, DC-81, PBD-conjugates PBD dimer and phosphonate–PBD conjugates.



Scheme 1. Reagents and conditions: (i) EtSH-BF_3OEt_2, CH_2Cl_2, 12 h, rt, 75%; (ii) diethyl(3-bromopropyl)phosphonate, K_2CO_3, acetone, reflux, 48 h, 80%; (iii) $SnCl_2$ $2H_2O$, MeOH, reflux, 4 h, 80%; (iv) $HgCl_2$–$CaCO_3$, CH_3CN: H_2O (4:1), 8–12 h, 60%.



Scheme 2. Reagents and conditions: (i) dibromoalkanes, K_2CO_3, acetone, reflux, 48 h, 90%; (ii) *n*-bocpiperazine, K_2CO_3, DMF, reflux, 36 h, 80%; (iii) TFA; CH_2Cl_2, rt, 8 h, 80%; (iv) Diethyl(2-bromoethyl)phosphonate, diethyl (2-bromo propyl)phosphonate, DMF, rt, 24 h, 60 %; (v) $SnCl_2$ $2H_2O$, MeOH, reflux, 4 h, 75%; (vi) $HgCl_2$–$CaCO_3$, CH_3CN: H_2O (4:1), 8–12 h, 60%.

upon incubation for 18 h. However, piparazine spacer plays major influence in these conjugates as good interaction with base pairs and proper snug fit in the minor groove of double stranded DNA.

2.3. RED_{100}-restriction endonuclease digestion assay

Several studies have employed restriction endonuclease inhibition to confirm the relative-binding affinity of DNA-interactive small molecule ligands.[33–35] A quantitative restriction enzyme digestion (RED_{100}) assay has been developed in which the inhibition of DNA cleavage by BamHI is used to probe the DNA-binding capability of PBD monomers.[36] We have earlier investigated this assay for preferences of base pair selectivity of the imine–amide PBD dimers.[20] Recently, this study has been carried out to determine the DNA-binding ability of benzothiadiazine–PBD conjugates.[37] Moreover, this technique has also been used to study the covalent DNA interaction of the PBD dimers and it is capable of distinguishing between the monomeric and dimeric families.[38] The drug was preincubated with DNA, the

Table 1. Thermal denaturation data of C8-linked phosphonate–PBD conjugates with CT-DNA

PBD conjugates	[PBD]:[DNA] molar ratio[a]	ΔT_m (°C)[b] after incubation at 37 °C for	
		0 h	18 h
6	1:5	2.1	4.5
7a	1:5	9.0	9.0
7b	1:5	9.0	9.9
7c	1:5	6.2	7.1
7d	1:5	6.1	7.2
7e	1:5	10.0	10.0
3	1:5	4.7	6.9
DC-81 (**2**)	1:5	0.3	0.7
PBD dimmer (**4**)	1:5	21.8	24.0

[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 = 69.2 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ±0.1–0.2 °C.

DNA–drug complexes were subjected to BamHI digestion, and the protection of cleavage of the G↓GATCC sequence by PBD suggests that these molecules selectively interact with G-rich sequences in DNA. The inability of PBD in protecting cleavage of AAT↓ATT by SspI suggests that these PBDs have low affinity to AT rich sequences. Therefore, PBD prefers G-rich sequences in DNA binding, and this could be due to the affinity of PBDs in covalent interaction with the free amino group attached to the N2 of guanine in the DNA.[36] The study has been carried out to determine the ability of **7a**, **7b**, **7d** and **7e**, which inhibited the DNA linearization by BamHI. The results of this experiment for compounds **7a**, **7b**, **7d** and **7e** have been shown in Fig. 2, suggesting that the phosphonate–PBDs inhibit BamHI. There have been differences in the inhibitory activity exhibited by PBDs evaluated in this assay. It is observed that ranking order is **7e** > **7b** > **7a** > **7d** for inhibition of BamHI cleavage. The BamHI inhibition of these conjugates has not correlated much with the DNA-binding affinity that may be due to the ability of piperazine moiety to interact with phosphonate backbone of DNA rather than to interact with the base pairs.

2.4. Cytotoxicity

Compounds **6**, **7d** and **7e** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines of breast, cervix, lung, colon, oral, ovarian and prostate by using Sulforhodamine B (SRB) method.[35,39] The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ M are considered to be active on the respective cell lines. Table 2 reveals that compounds **6**, **7d** and **7e** have exhibited significant effect against some of cell lines and the activity was comparable with adriamycin (**ADR**). Interestingly, the piperazine containing compound **7d** exhibited a strong effect in terms of GI_{50} values on SiHa (0.21 μM), GURAV (0.17 μM) and A2780 (0.17 μM) cell lines and the compound **7e** exhibited GI_{50} value to the order of 0.18 μM against DWD cell line. The comparison of the data in Table 2 reveals that absence of piperazine spacer in these PBD conjugates moderately decreases cytotoxic activity or shows no activity in some of the cell lines (**6**). However, the in vitro cytotoxicity (GI_{50}) for naturally occurring DC-81[16] is 0.38 and 0.33 μM in L1210 and PC-3 cell lines.

The compound **7d** has been selected for NCI-60 cell line panel, and comparison of the data in Table 3 reveals the average $\log_{10}GI_{50}$, $\log_{10}$ TGI and $\log_{10}$ LC_{50} values of the cytotoxic activity in 60 human cancer cell lines in nine panels and each panel containing average of six to eight human cancer cell lines. Interestingly the compound **7d** possesses selective anticancer activity particularly for leukemia. The leukemia cancer values ($\log_{10}$ GI_{50}, $\log_{10}$ TGI and $\log_{10}$ LC_{50}) for the compound (**7d**) have been described in Table 4.

Moreover, the mean graph mid point values of $\log_{10}$ TGI and $\log_{10}$ LC_{50} as well as $\log_{10}$ GI_{50} for **5** and **7d**

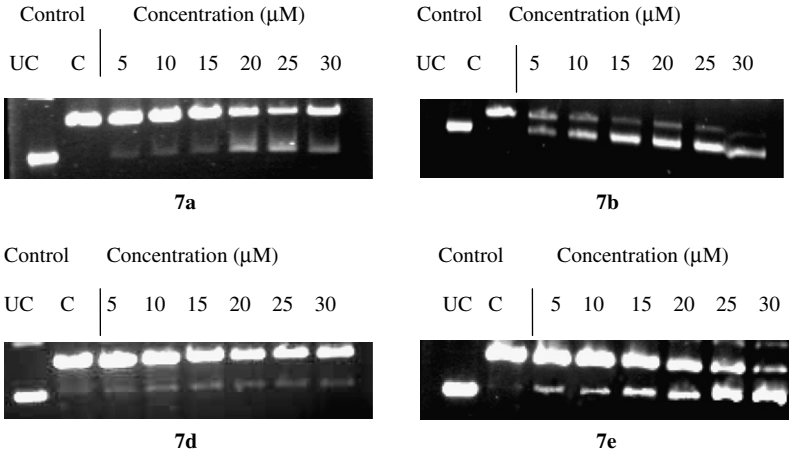


Figure 2. RED_{100}-restriction endonuclease digestion assay for PBD–phosphonate conjugates with CT-DNA inhibitory activity of **7a**, **7b**, **7d** and **7e** on the cleavage of plasmid pBR322 by restriction endonuclease BamHI (10 U in 1 μL) for 1 h at 37 °C. The cut (C) and uncut (UC) products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination. Lane 1, control pBR322; lane 2, complete digest of pBR322 by BamHI.

Table 2. GI_{50} values[a] (in μM) for compounds **6**, **7d** and **7e** tested in selected human malignant cell lines

Cell lines	6	7d	7e	ADR
MCF7[b]	2.02	2.01	2.21	0.18
SiHa[c]	2.10	0.21	2.46	0.18
A-549[d]	[i]	2.64	[i]	7.25
Colo205[e]	[i]	1.87	2.39	0.14
KB[f]	2.11	1.92	[i]	0.17
GURAV[f]	[i]	0.17	2.18	0.17
DWD[f]	2.20	[i]	0.18	0.11
A2780[g]	[i]	0.17	2.02	0.17
PC-3[h]	[i]	1.98	30.5	1.81

[a] Obtained from three determinations.
[b] Breast cancer.
[c] Cervix cancer.
[d] Lung cancer.
[e] Colon cancer.
[f] Oral cancer.
[g] Ovarian cancer.
[h] Prostate cancer.
[i] GI_{50} value not attained at the concentrations used in the assay; ADR, adriamycin.

Table 3. $\log_{10}$ GI_{50}, $\log_{10}$ TGI and $\log_{10}$ LC_{50} (concentration in mol/L) values for the representative compound **7d**

Cancer panel	$\log_{10}$ GI_{50}	$\log_{10}$ TGI	$\log_{10}$ LC_{50}
Leukemia	−5.71	−4.91	−4.00
Lung	−5.00	−4.34	−4.00
Colon	−5.00	−4.33	−4.03
CNS	−5.52	−4.52	−4.05
Melanoma	−5.11	−4.52	−4.09
Ovarian	−4.86	−4.26	−4.00
Renal	−5.01	−4.27	−4.02
Prostate	−4.75	−4.20	−4.00
Breast	−5.25	−4.48	−4.02

Each cancer type represents the average of six to nine different cancer cell lines.

Table 4. The leukemia cancer values for the representative compound **7d**

Leukemia	Log GI_{50}	Log TGI	Log LC_{50}
CCRF-CEM	−5.72	nd[a]	>−4.00
HL-60(TB)	−5.74	−5.08	>−4.00
K-562	−5.63	>−4.00	>−4.00
MOLT-4	−5.67	−5.20	>−4.00
RPMI-8226	−5.64	nd	>−4.00
SR	−5.86	−5.37	−4.51

[a] nd = not determined.

Table 5. $\log_{10}$ GI_{50} $\log_{10}$ TGI and $\log_{10}$ LC_{50} mean graph midpoints (MG_MID) of in vitro cytotoxicity data for the compounds **5** and **7d** against human tumour cell lines

Compound	$\log_{10}$ GI_{50}	$\log_{10}$ TGI	$\log_{10}$ LC_{50}
7d	−5.14	−4.42	−4.04
5[a]	−4.62	−4.25	−4.07

[a] See Ref. 26.

are listed in Table 5. As demonstrated by mean graph pattern compound **7d** exhibits an interesting profile of activity and selectivity for various cell lines. Therefore, the cytotoxicity activities exhibited by these new PBD conjugates are significant.

3. Conclusion

In conclusion, C8-linked piperazine-containing phosphonate–PBD conjugates have been synthesized that exhibit remarkable DNA-binding ability. The restriction endonuclease study also demonstrates this aspect and suggests that these molecules selectively interact with G-sequences in DNA and low affinity with AT-rich sequences. More importantly, inclusion of a piperazine moiety in the linker spacer not only enhances the potency of such PBD conjugates but also their solubility profile. Moreover, **7d** exhibits significant cytotoxicity in some of the cancer cell lines and these investigations will also be helpful in the design of improved DNA targeted molecules.

4. Experimental

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 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), and m (multiplet). Coupling constants are reported in Hertz (Hz). EI mass spectra were recorded on a VG-7070H Micromass mass spectrometer at 200 °C, 70 eV, with a trap current of 200 μA and 4 kV of acceleration voltage. ESI spectra were recorded on Micro mass, Quattro LC using ESI^+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High-resolution mass spectra were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. FAB mass spectra were recorded on a LSIMS-VG-AUTOSPEC Micromass spectrometer. Micro analytical data (C, H and N) agreed with the proposed structures within ±0.4% of the theoretical values.

5. General procedure

5.1. Synthesis of (2*S*)-*N*-(4-hydroxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxal-dehyde diethyl thioacetal (*9*)

To a stirred solution of EtSH (1.91 g, 19.0 mmol) and $BF_3.OEt_2$ (1.41 g, 10 mmol) was added dropwise to the compound **8** (0.490 g, 1 mmol) in dichloromethane (10 mL) at room temperature. Stirring was continued un-

til TLC indicated completion of the reaction. The solvent was evaporated in vacuum. The residue was quenched with bicarbonate solution (1 × 25 mL) and then extracted with ethyl acetate (3 × 25 mL). The combined organic phase was washed with saturated brine (1 × 25 mL) dried over Na_2SO_4 and the solvent removed in vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate–hexane (7:3) as eluent to afford compound **9** (0.30 g, 75%).

^{1}H NMR ($CDCl_3$): δ 7.65 (s, 1H), 6.75 (s, 1H), 6.20–6.32 (br s, 1H), 4.85 (d, 1H), 4.60–4.70 (m, 1H), 3.95 (s, 3H), 3.20–3.32 (m, 2H), 2.70–2.88 (m, 4H), 1.75–2.35 (m, 4H), 1.20–1.40 (m, 6H); MS (EI) 400 $[M]^+$; Anal. Calcd for $C_{17}H_{24}N_2O_5S_2$: C, 50.98; H, 6.04; N, 6.99. Found: C, 50.63; H, 5.98; N, 6.58.

5.2. Synthesis of (2*S*)-*N*-{4-[3-(diethoxyphosphoryl)propyl]oxy-5-methoxy-2-nitrobenzo-yl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (10)

To a solution of compound **9** (400 mg, 1 mmol) in dry acetone (15 mL) were added anhydrous K_2CO_3 (553 mg, 4 mmol), and diethyl(3-bromopropyl)phosphonate (310 mg, 1.2 mmol) and the mixture was stirred at reflux temperature for 48 h. The reaction was monitored by TLC using (95% EtOAc–methanol), K_2CO_3 was removed by filtration and the solvent was evaporated under the vacuum, diluted with water and extracted with ethyl acetate. The combined organic phase dried (Na_2SO_4) and evaporated in vacuum was purified by column chromatography (3% EtOAc–methanol) to afford compound **10** as yellow liquid (0.47 g, 80%).

^{1}H NMR ($CDCl_3$): δ 7.63 (s, 1H), 6.77 (s, 1H), 4.82 (d, J = 3.7 Hz, 1H), 4.60–4.70 (m, 1H), 4.05–4.20 (m, 6H), 3.94 (s. 3H), 3.16–3.32 (m, 2H), 2.65–2.85 (m, 4H), 1.80–2.20 (m, 8H), 1.20–1.40 (m, 12H); MS (ESI) 593 $[M+1]^+$; Anal. Calcd for $C_{24}H_{39}N_2O_8PS_2$: C, 49.81; H, 6.79; N, 4.84. Found: C, 49.86; H, 6.75; N, 4.82.

5.3. Synthesis of (2*S*)-*N*-4-[3-(diethoxyphosphoryl)propyl]oxy-5-methoxy-2-aminobenzo-yl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (11)

The compound **10** (593 mg, 1 mmol) dissolved in methanol (20 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) was refluxed for 4 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 ethyl acetate (2 × 30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **11** as brown oil (0.43 g, 80%) which due to potential stability problems was directly used in the next step without isolation.

5.4. Synthesis of 7-methoxy-8-[3-(diethoxyphosphoryl)propyl]oxy-(11a*S*)-1,2,3,11a-tetra- hydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (6)

A solution of **11** (549 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in MeCN–water (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material. The reaction mixture was diluted with EtOAc (30 mL) and filtered through a celite bed. The clear yellow 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 in vacuum and purified by column chromatography (80% CH_2Cl_2–MeOH) to give compound **6** (0.25 g, 60%). This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

^{1}H NMR ($CDCl_3$): δ 7.70 (s, 1H); 7.65 (d, J = 4.4 Hz, 1H), 6.80 (s, 1H), 4.62–4.75 (m, 1H), 4.10–4.30 (m, 6H), 3.90 (s, 3H), 3.50–3.70 (m, 2H), 1.76–2.39 (m, 8H), 1.25–1.40 (m, 6H); MS (ESI) 424 $[M]^+$; Anal. Calcd for $C_{20}H_{29}N_2O_6P$: C, 49.81; H, 6.79; N, 4.84. Found: C, 49.86; H, 6.75; N, 4.82.

5.5. General procedure for the synthesis of compounds (12a–c)

5.5.1. (2*S*)-*N*-[4-(3-Bromopropyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxal-dehyde diethyl thioacetal (12a). To a stirred solution of (2*S*)-*N*-(4-hydroxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal (**9**) (0.40 g, 1 mmol) in acetone (15 mL) were added anhydrous potassium carbonate (0.69 g, 5.0 mmol) and compound 1,3-dibromopropane (0.80 g, 4 mmol). The reaction mixture was refluxed in an oil bath for 48 h and 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 solvent was removed under vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate–hexane (8:2) as a solvent system to give product **12a** (0.46 g, 90%).

^{1}H NMR ($CDCl_3$): δ, 7.70 (s, 1H), 6.80 (s, 1H), 4.85 (d, J = 4.3 Hz, 1H), 4.63–4.75 (m, 1H), 4.25 (t, J = 5.98 Hz, 2H), 3.95 (s, 3H), 3.65 (t, J = 6.22 Hz, 2H), 3.20–3.35 (m, 2H), 2.60–2.90 (m, 4H), 1.70–2.50 (m, 6H), 1.20–1.40 (m, 6H); MS (FAB) 521 $[M]^+$; Anal. Calcd for $C_{20}H_{29}BrN_2O_5S_2$: C, 46.06; H, 5.61; N, 5.37. Found: C, 46.00; H, 5.65; N, 5.35.

5.5.2. (2*S*)-*N*-[4-(4-Bromobutyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxalde-hyde diethyl thioacetal (12b). The compound **12b** was prepared according to the method described for compound **12a** employing (2*S*)-*N*-(4-hydroxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal (**9**) (535 mg, 1 mmol) and 1,4-dibromobutane (0.86 g, 4 mmol) to afford compound **12b** (0.48 g, 90%).

^{1}H NMR ($CDCl_3$): δ 1.21–1.45 (m, 6H), 1.70–2.40 (m, 8H), 2.60–2.90 (m, 4H), 3.15–3.30 (m, 2H), 3.50 (t, J = 6.25 Hz, 2H), 3.95 (s, 3H), 4.10 (t, J = 6 Hz, 2H), 4.60–4.71 (m, 1H), 4.85 (d, J = 4.3 Hz, 1H), 6.80 (s, 1H), 7.65 (s, 1H); MS (FAB) 535 $[M]^+$; Anal. Calcd for $C_{21}H_{31}BrN_2O_5S_2$: C, 47.10; H, 5.83; N, 5.23. Found: C, 47.15; H, 5.82; N, 5.26.

5.5.3. (2*S*)-*N*-[4-(5-Bromopentyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxal-dehyde diethyl thioacetal (12c). The compound **12c** was prepared according to the method described for compound **12a** employing (2*S*)-*N*-(4-hydroxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal (**9**) (535 mg, 1 mmol) and 1,5-dibromopentane (0.92 g, 4 mmol) to afford compound **12c** (0.49 g, 90%).

^{1}H NMR ($CDCl_3$): δ 7.65 (s, 1H); 6.80 (s, 1H), 4.85 (d, J = 4.33 Hz, 1H), 4.60–4.71 (m, 1H), 4.10 (t, J = 6 Hz, 2H), 3.95 (s, 3H), 3.45 (t, J = 6.34 Hz, 2H), 3.15–3.30 (m, 2H), 2.65–2.85 (m, 4H), 1.60–2.40 (m, 8H), 1.21–1.40 (m, 8H); MS (FAB) 549 $[M]^+$; Anal. Calcd for $C_{22}H_{33}BrN_2O_5S_2$: C, 48.08; H, 6.05; N, 5.10. Found: C, 48.09; H, 6.02; N, 5.11.

5.6. General procedure for the synthesis of compounds (13a–c)

5.6.1. (2*S*)-*N*-4-[3-[(4^1-*tert*-Butoxycarbonyl)piperazin-1-yl]propyl]oxy-5-methoxy-2-nitro benzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (13a). To a stirred solution of *n*-bocpiperazine (186 mg, 1 mmol) in dry DMF (10 mL) were added anhydrous potassium carbonate (0.69 g, 5.0 mmol) and compound (2*S*)-*N*-[4(3-bromopropyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**12a**) (0.62 g, 1.2 mmol). The reaction mixture was stirred at room temperature for 36 h and the reaction was monitored by TLC using (4% ethyl acetate–methanol) as a solvent system. The potassium carbonate was removed by suction filtration and solvent was removed under vacuum, diluted with water and extracted with ethyl acetate. The combined organic phase was dried (Na_2SO_4) and evaporated in vacuum and it was purified by column chromatography (3% EtOAc–methanol) to afford compound **13a** as brown oil (0.50 g, 80%).

^{1}H NMR ($CDCl_3$): δ 7.71 (s, 1H), 6.83 (s, 1H), 4.87–4.89 (d, J = 3.7 Hz, 1H), 4.68–4.76 (m, 1H), 4.18 (t, J = 6.0 Hz, 2H), 3.94 (s, 3H), 3.40–3.50 (t, J = 4.5 Hz, 4H), 3.20–3.32 (m, 2H), 2.70–2.85 (m, 4H), 2.52–2.60 (t, J = 6.7 Hz, 2H), 2.38–2.46 (t, J = 4.5 Hz, 4H), 2.03–2.15 (m, 4H), 1.60–1.70 (m, 2H), 1.46 (s, 9H), 1.30–1.39 (q, 6H); MS (ESI) 627 $[M+1]^+$; Anal. Calcd for $C_{29}H_{46}N_4O_7S_2$: C, 55.57; H, 7.40; N, 8.94. Found: C, 55.51; H, 7.43; N, 8.95.

5.6.2. (2*S*)-*N*-4-[4-[(4^1-*tert*-Butoxycarbonyl)piperazin-1-yl]butyl]oxy-5-methoxy-2-nitro benzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (13b). The compound **13b** was prepared according to the method described for compound **13a** employing *n*-bocpiperazine (186 mg, 1 mmol) and (2*S*)-*N*-[4-(4-bromobutyl)oxy-5-methoxy-2 nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**12b**) (0.64 g, 1.2 mmol) to afford compound **13b** (0.51 g, 80%).

^{1}H NMR ($CDCl_3$): δ 7.66 (s, 1H), 6.77 (s, 1H), 4.80–4.82 (d, J = 6.82 Hz, 1H), 4.61–4.70 (m, 1H), 4.12–4.18 (t, J = 6.82 Hz, 2H), 3.95 (s, 3H), 3.40–3.49 (m, 4H), 3.19–3.30 (m, 2H), 2.68–2.88 (m, 4H), 2.52–2.58 (t, J = 6.82 Hz, 2H), 2.42–2.50 (m, 4H), 2.32–2.38 (m, 4H), 2.10–2.20 (m, 4H), 1.47 (s, 9H), 1.30–1.42 (m, 6H); MS (ESI) 641 $[M+1]^+$; Anal. Calcd for $C_{30}H_{48}N_4O_7S_2$: C, 56.23; H, 7.55; N, 8.74. Found: C, 56.27; H, 7.56; N, 8.70.

5.6.3. (2*S*)-*N*-4-[5-[(4^1-*tert*-Butoxycarbonyl)piperazin-1-yl]pentyl]oxy-5-methoxy-2-nitro benzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (13c). The compound **13c** was prepared according to the method described for compound **13a** employing *n*-bocpiperazine (186 mg, 1 mmol) and (2*S*)-*N*-[4-(5-bromopentyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**12c**) (0.65 g, 1.2 mmol) to afford compound **13c** (0.52 g, 80%).

^{1}H NMR ($CDCl_3$): δ 7.66 (s, 1H), 6.77 (s, 1H), 4.81 (d, J = 6.51 Hz, 1H), 4.61–4.70 (m, 1H), 4.15 (t, J = 6.51 Hz, 2H), 3.95 (s, 3H), 3.39–3.47 (m, 4H), 3.17–3.30 (m, 2H), 2.66–2.87 (m, 4H), 2.54 (t, J = 6.84 Hz, 2H), 2.40–2.48 (m, 4H), 2.30–2.38 (m, 4H), 1.92–2.14 (m, 6H), 1.46 (s, 9H), 1.31–1.40 (m, 6H); MS (ESI) 655 $[M+1]^+$; Anal. Calcd for $C_{31}H_{50}N_4O_7S_2$: C, 56.86; H, 7.70; N, 8.56. Found: C, 56.82; H, 7.74; N, 8.53.

5.7. General procedure for the synthesis of compounds (14a–c)

5.7.1. (2*S*)-*N*-{4-[3-piperazinopropoxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (14a). To a solution of boc-protected compound **13a** (627 mg, 1 mmol) in dry dichloromethane was added trifluoroacetic acid (1 mL) at 0 °C and stirred under nitrogen for 8 h, the reaction mixture was concentrated in vacuum and then it was used directly in the next step.

5.7.2. (2*S*)-*N*-{4-[4-piperazinobutoxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (14b). The compound **14b** was prepared according to the method described for compound **14a** employing boc-protected compound **13b** (641 mg, 1 mmol) and trifluoroacetic acid (1 mL) to afford compound **14b**.

5.7.3. (2*S*)-*N*-{4-[5-piperazinopentoxy]-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (14c). The compound **14c** was prepared according to the method described for compound **14a** employing boc-protected compound **13c** (655 mg, 1 mmol) and trifluoroacetic acid (1 mL) to afford compound **14c**.

5.8. General procedure for the synthesis of compounds (15a–e)

5.8.1. (2*S*)-*N*-{4-[3-[4-[2-(Diethoxyphosphoryl)ethyl]piperazin-1-yl]propyl]-oxy-5-meth-oxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (15a). To a stirred solution of (2*S*)-*N*-[4-(3-piperazinopropyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**14a**) (441 mg, 1 mmol) in dry DMF (10 mL) were added anhydrous potassium carbonate (0.69 g, 5.0 mmol) and compound diethyl(2-bromoethyl)phosphonate (294 mg, 1.2 mmol). The reaction

mixture was stirred at room temperature for 24 h and the reaction was monitored by TLC using (10% CH_2Cl_2–methanol) as a solvent system. The potassium carbonate was removed by suction filtration and solvent was removed under vacuum, diluted with water and extracted with ethyl acetate. The combined organic phase dried (Na_2SO_4) and evaporated in vacuum was purified by column chromatography (5% CH_2Cl_2–methanol) to afford compound **15a** as brown oil (0.45 g, 60%).

^{1}H NMR ($CDCl_3$): δ 7.61 (s, 1H), 6.78 (s, 1H), 4.82 (d, 1H, J = 3.5 Hz), 4.62–4.72 (m, 1H), 4.00–4.20 (m, 6H), 3.94 (s, 3H), 3.15–3.31 (m, 2H), 2.50–2.90 (m, 16H), 1.44–2.40 (m, 8H), 1.15–1.40 (m, 12H); MS (ESI) 537 $[M+1]^+$; Anal. Calcd for $C_{30}H_{51}N_4O_8PS_2$: C, 52.16; H, 7.44; N, 8.11. Found: C, 52.12; H, 7.40; N, 8.09.

5.8.2. (2*S*)-*N*-{4-[3-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (15b). The compound **15b** was prepared according to the method described for compound **15a** employing (2*S*)-*N*-[4-(3-piperazinopropyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**14a**) (441 mg, 1 mmol) and diethyl(3-bromopropyl)phosphonate (310 mg, 1.2 mmol) to afford compound **15b** (0.46 g, 65%).

^{1}H NMR ($CDCl_3$): δ 7.66 (s, 1H), 6.77 (s, 1H), 4.82 (d, J = 3.9 Hz, 1H), 4.63–4.70 (m, 1H), 4.00–4.20 (m, 7H), 3.94 (s, 3H), 3.16–3.30 (m, 2H), 2.65–2.90 (m, 4H), 2.40–2.65 (m, 12H), 1.40–2.40 (m, 10H), 1.28–1.38 (m, 12H); MS (ESI) 719 $[M+1]^+$; Anal. Calcd for $C_{31}H_{53}N_4O_8PS_2$: C, 52.82; H, 7.58; N, 7.95. Found: C, 52.79; H, 7.60; N, 7.98.

5.8.3. (2*S*)-*N*-{4-[4-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]butyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (15c). The compound **15c** was prepared according to the method described for compound **15a** employing (2*S*)-*N*-[4-(4-piperazinobutyl)oxy]-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**14b**) (455 mg, 1 mmol) and diethyl(3-bromopropyl)phosphonate (310 mg, 1.2 mmol) to afford compound **15c** (0.47 g, 65%).

^{1}H NMR ($CDCl_3$): δ 7.61(s, 1H), 6.77 (s, 1H), 4.82 (d, J = 3.7 Hz, 1H), 4.61–4.71 (m, 1H), 4.00–4.16 (m, 6H), 3.94 (s, 3H), 3.15–3.30 (m, 2H), 2.65–2.85 (m, 4H), 2.35–2.60 (m, 12H), 1.40–2.40 (m, 12H), 1.20–1.40 (m, 12H); MS (ESI) 733 $[M+1]^+$; Anal. Calcd for $C_{32}H_{55}N_4O_8PS_2$: C, 53.46; H, 7.71; N, 7.79. Found: C, 53.41; H, 7.76; N, 7.81.

5.8.4. (2*S*)-*N*-{4-[5-[4-[2-(Diethoxyphosphoryl)ethyl]piperazin-1-yl]pentyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (15d). The compound **15d** was prepared according to the method described for compound **15a** employing (2*S*)-*N*-[4-(5-piperazinopentyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**14c**) (469 mg, 1 mmol) and diethyl(2-bromoethyl)phosphonate (294 mg, 1.2 mmol) to afford compound **15d** (0.47 g, 65%).

^{1}H NMR ($CDCl_3$): δ 7.62 (s, 1H), 6.75 (s, 1H), 4.80 (d, J = 3.6 Hz, 1H), 4.60–4.71 (m, 1H), 4.04–4.16 (m, 6H), 3.92 (s, 3H), 3.15–3.30 (m, 2H), 2.65–2.85 (m, 4H), 2.35–2.60 (m, 12H), 1.42–2.33 (m, 10H), 1.20–1.42 (m, 12H), 0.82–0.92 (m, 2H); MS (ESI) 733 $[M+1]^+$; Anal. Calcd for $C_{32}H_{55}N_4O_8PS_2$: C, 53.46; H, 7.71; N, 7.79. Found: C, 53.41; H, 7.76; N, 7.81.

5.8.5. (2*S*)-*N*-{4-[5-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]pentyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (15e). The compound **15e** was prepared according to the method described for compound **15a** employing (2*S*)-*N*-[4-(5-piperazinopentyl)oxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**14c**) (469 mg, 1 mmol) and diethyl(3-bromopropyl)phosphonate (310 mg, 1.2 mmol) to afford compound **15e** (0.48 g, 65%).

^{1}H NMR ($CDCl_3$): δ 7.61 (s, 1H), 6.77 (s, 1H), 4.82 (d, J = 3.7 Hz, 1H), 4.61–4.71 (m, 1H), 4.00–4.16 (m, 6H), 3.94 (s, 3H), 3.15–3.30 (m, 2H), 2.65–2.85 (m, 4H), 2.35–2.60 (m, 12H), 1.42–2.33 (m, 12H), 1.20–1.40 (m, 12H), 0.84–0.92 (m, 2H); MS (ESI) 747 $[M+1]^+$; Anal. Calcd for $C_{33}H_{57}N_4O_8PS_2$: C, 54.08; H, 7.84; N, 7.64. Found: C, 54.12; H, 7.88; N, 7.60.

5.9. General procedure for the synthesis of compounds (16a–e)

5.9.1. (2*S*)-*N*-{4-[3-[4-[2-(Diethoxyphosphoryl)ethyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (16a). The compound **15a** (705 mg, 1 mmol) dissolved in methanol (20 mL) and added $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) was refluxed for 4 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 ethyl acetate (2×30 mL). The combined organic phase was dried over Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal **16a** as brown oil (0.50 g, 75%) which due to potential stability problems was directly used in the next step without isolation.

5.9.2. (2*S*)-*N*-{4-[3-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (16b). The compound **16b** was prepared according to the method described for compound **16a** employing (2*S*)-*N*-{4-[3-[4-[3-(diethoxyphosphoryl)propyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**15b**) (469 mg, 1 mmol) and $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) to afford compound **16b** (0.51 g, 75%), which due to potential stability problems was directly used in the next step without isolation.

5.9.3. (2*S*)-*N*-{4-[4-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]butyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (16c). The compound **16c** was prepared according to the method described for compound **16a** employing (2*S*)-*N*-{4-[3-[4-[4-(diethoxyphosphoryl)butyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-nitro- benzoyl}pyrrolidine-2-carboxal-

dehyde diethyl thioacetal (**15c**) (469 mg, 1 mmol) and $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) to afford compound **16c** (0.52 g, 75%), which due to potential stability problems was directly used in the next step without isolation.

5.9.4. (2*S*)-*N*-{4-[3-[4-[2-(Diethoxyphosphoryl)ethyl]piperazin-1-yl]pentyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (16d). The compound **16d** was prepared according to the method described for **16a** employing (2*S*)-*N*-{4-[3-[4-[4-(diethoxyphosphoryl)pentyl]piperazin-1-yl]ethyl]-oxy-5-methoxy-2-nitro-benzoy l}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**15d**) (469 mg, 1 mmol) and $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) to afford compound **16d** (0.52 g, 75%), which due to potential stability problems was directly used in the next step without isolation.

5.9.5. (2*S*)-*N*-{4-[3-[4-[3-(Diethoxyphosphoryl)propyl]piperazin-1-yl]pentyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (16e). The compound **16e** was prepared according to the method described for compound **16a** employing (2*S*)-*N*-{4-[3-[4-[4-(diethoxyphosphoryl)pentyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-nitro-benzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**15e**) (469 mg, 1 mmol) and $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) to afford compound **16e** (0.53 g, 75%), which due to potential stability problems was directly used in the next step without isolation.

5.10. General procedure for the synthesis of compounds (7a–e)

5.10.1. 7-Methoxy-8-[3-[4-[2-(diethoxyphosphoryl)ethyl]piperazin-1-yl]propyl]oxy-(11a-*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7a). A solution of **16a** (675 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) in MeCN–water (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material. The reaction mixture was diluted with EtOAc (30 mL) and filtered through a celite bed. The clear yellow organic supernatant was extracted with saturated 5% $NaHCO_3$ (20 mL), and brine (20 mL) and the combined organic phase was dried (Na_2SO_4). The organic layer was evaporated in vacuum and purified by column chromatography (80% CH_2Cl_2–MeOH) to give compound **7a** (0.32 g, 60%). This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form.

^{1}H NMR ($CDCl_3$): δ 7.57 (d, J = 4.4 Hz, 1H), 7.50 (s, 1H), 6.83 (s, 1H), 4.00–4.20 (m, 7H), 3.92 (s, 3H), 3.50–3.88 (m, 2H), 2.40–2.80 (m, 12H), 2.20–2.40 (m, 2H), 1.80–2.15 (m, 6H), 1.20–1.36 (m, 6H); MS (ESI) 536 $[M+1]^+$; Anal. Calcd for $C_{26}H_{41}N_4O_6P$: C, 58.20; H, 7.70; N, 10.44. Found: C, 58.25; H, 7.72; N, 10.40.

5.10.2. 7-Methoxy-8-[3-[4-[3-(diethoxyphosphoryl)propyl]piperazin-1-yl]propyl]oxy-(11a-*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7b). The compound **7b** was prepared according to the method described for compound **7a** employing **16b** (689 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to afford compound **7b** (0.33 g, 68%).

^{1}H NMR ($CDCl_3$): δ 7.69 (d, J = 4.4 Hz, 1H), 7.56 (s, 1H), 6.88 (s, 1H), 4.60–4.88 (m, 1H), 4.00–4.28 (m, 6H), 3.96 (s, 3H), 3.48–3.92 (m, 2H), 2.24–2.70 (m, 12H), 2.00–2.24 (m, 2H), 1.60–1.96 (m, 8H), 1.20–1.40 (m, 6H); MS (ESI) 564 $[M+1]^+$; Anal. Calcd for $C_{27}H_{43}N_4O_6P$: C, 58.90; H, 7.87; N, 10.18. Found: C, 58.92; H, 7.85; N, 10.22.

5.10.3. 7-Methoxy-8-[4-[4-[3-(diethoxyphosphoryl)propyl]piperazin-1-yl]butyl]oxy-(11a*S*) 1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7c). The compound **7c** was prepared according to the method described for compound **7a** employing **16c** (703 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to afford compound **7 c** (0.34 g, 68%).

^{1}H NMR ($CDCl_3$): δ 7.65 (d, J = 4.4 Hz, 1H), 7.55 (s, 1H), 6.88 (s, 1H), 4.58–4.88 (m, 1H), 4.04–4.28 (m, 6H), 3.95 (s, 3H), 3.48–3.92 (m, 2H), 2.25–2.72 (m, 12H), 2.02–2.24 (m, 2H), 1.60–1.96 (m, 10H), 1.20–1.40 (m, 6H); MS (ESI) 578 $[M+1]^+$; Anal. Calcd for $C_{28}H_{45}N_4O_6P$: C, 59.56; H, 8.03; N, 9.92. Found: C, 59.58; H, 8.00; N, 9.92.

5.10.4. 7-Methoxy-8-[5-[4-[2-(diethoxyphosphoryl)ethyl]piperazin-1-yl]pentyl]oxy-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7d). The compound **7d** was prepared according to the method described for compound **7a** employing **16d** (703 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to afford compound **7d** (0.34 g, 68%).

^{1}H NMR ($CDCl_3$): δ 7.67 (d, J = 3.9 Hz, 1H), 7.50 (s, 1H), 6.80 (s, 1H), 4.00–4.20 (m, 7H), 3.87 (s, 3H), 3.42–3.82 (m, 2H), 1.80–2.80 (m, 16H), 1.20–1.72 (m, 8H), 0.80–0.96 (m, 2H); MS (ESI) 578 $[M+1]^+$; Anal. Calcd for $C_{28}H_{45}N_4O_6P$: C, 59.56; H, 8.03; N, 9.92. Found: C, 59.58; H, 8.00; N, 9.92.

5.10.5. 7-Methoxy-8-[5-[4-[3-(diethoxyphosphoryl)propyl]piperazin-1-yl]pentyl]oxy-(11a-*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (7e). The compound **7e** was prepared according to the method described for compound **7a** employing **16e** (717 mg, 1 mmol), $HgCl_2$ (613 mg, 2.26 mmol) and $CaCO_3$ (246 mg, 2.46 mmol) to afford compound **7e** (0.36 g, 68%).

^{1}H NMR ($CDCl_3$): δ 7.66 (d, J = 4.0 Hz, 1H,), 7.52 (s, 1H, Ar), 6.82 (s, 1H), 4.02–4.22 (m, 7H), 3.86 (s, 3H), 3.40–3.82 (m, 2H), 1.80–2.80 (m, 16H), 1.20–1.76 (m, 10H), 0.82-0.98 (m, 2H); MS (ESI) 592 $[M+1]^+$; Anal. Calcd for $C_{29}H_{47}N_4O_6P$: C, 60.19; H, 8.19; N, 9.68. Found: C, 60.22; H, 8.25; N, 9.62.

6. Biological activity

6.1. Thermal denaturation studies

The DNA-binding affinity of the novel phosphonate–PBD conjugates (**6** and **7a**–**e**) and *N*-methylpiperazinyl–

PBD conjugate (**3**) has been evaluated through thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using modified reported procedure.[31] 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 MeOH to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions were incubated at 37 °C for 0, and 18 h prior to analysis. Samples were monitored at 260 nm using a Beckman DU-7400 spectrophotometer fitted with high-performance temperature controller, and heating was applied at 1 °C min^{-1} in the 40–90 °C range. DNA helix coil transition temperatures (T_m) were obtained from the maxima in the (dA260)/dT derivative plots. Results are given as means ± standard deviation from three determinations and are corrected for the effects of MeOH co-solvent using a linear correction term.[32] Drug-induced alterations in DNA melting behaviour are given by $\Delta T_m = T_m(\text{DNA + PBD}) - T_m$ (DNA alone), where the T_m value for the PBD-free CT-DNA is 69.2 ± 0.01. The fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

6.2. In vitro evaluation of cytotoxic activity

In-routine compounds **6**, **7d** and **7e** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines of breast (Zr-75-1, MCF7), cervix (SiHa), lung (Hop62, A-549), colon (Colo205), oral (KB, GURAV, DWD), ovarian (A2780), and prostate (PC-3) 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.[40,41]

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.

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 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–by-plate 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) × 100.

6.3. Restriction endonuclease inhibition

Stock solutions of each PBD (100 μM) were prepared by dissolving each compound in DMSO (Sigma). These were stored at −20 °C. Plasmid (pBR 322) containing single BamHI site was used in this assay. Restriction endonuclease and the relevant buffer were obtained from NEB. The DNA fragment (500 ng) was incubated with each PBD (see Fig. 2 for PBD concentrations) in a final volume of 16 μL for 16 h at 37 °C. Next 10 × BamHI buffer (2 μL) was added, and the reaction mixture was made up to 20 μL with BamHI (20 U) and then incubated for 1 h at 37 °C. Then loaded on to a 1% agarose gel electrophoresis in Tris–acetate EDTA buffer at 80 V for 2 h. The gels were stained with ethidium bromide and photographed.

Acknowledgments

The authors P.P.K., B.N.S., O.S and M.S.K. thank CSIR, New Delhi for the award of research fellowships. We are also thankful to the Department of Biotechnology (BT/PR/7037/Med/14/933/2006), New Delhi for financial assistance.

References and notes

1. Noyori, R. *Asymmetric Catalysis in Organic Synthesis*; John Wiley & Sons: New York, 1994.
2. Broch, R. F.; Canute, G. W. *J. Med. Chem.* **1991**, *34*, 3044.
3. Pawelczyk, E.; Zajac, M. *Chemiczne mechanizmy dzialania lek'ow*, (Akademia Medyczna in Poznan, **1995**, pp 364–377.
4. Kafarski, P.; Lejczak, B. *Curr. Med. Chem. Anticancer Agents* **2001**, *1*, 301.
5. Atherton, F. R.; Hassall, C. H.; Lambert, R. W. *J. Med. Chem.* **1986**, *29*, 29.
6. Smith, W. W.; Bartlett, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 4622.
7. Budzisz, E.; Wojciechowska, J. G.; Ziebab, R.; Nawrotc, B. *New J. Chem* **2002**, *26*, 1799.
8. Troev, K.; Botisov, G. *Phosphorus, Sulfur Silicon* **1987**, *29*, 129.
9. Zech, J. D.; U.S. Patent 2824113, 1958; *Chem. Abstr.* **1958**, *52*, 11110.
10. Chmiel, A.; Grudzinski, S. *Biotechnologia i Chemia Antybiotyk ow*, WN PWN, Warszawa, Ed.; 1998, pp 280.
11. Budzisz, E.; Brzezinska, E.; Krajewsk, U.; Rozalski, M. *Eur. J. Med. Chem.* **2003**, *38*, 597.
12. Kostka, K.; Pastuszko, S.; Porada, M. *Phosphorus, Sulfur Silicone* **1992**, *71*, 67.
13. Kramata, P.; Downey, K. M. *Mol. Pharmacol.* **1999**, *56*, 1262.
14. Lavielle, G.; Hautefaye, P.; Schaeffer, C.; Boutin, J. A.; Claude, A.; Cudennec; Pierr, A. *J. Med. Chem.* **1991**, *34*, 1998.
15. Thurston, D. E. *Molecular Aspects of Anticancer Drug–DNA Interactions*; The Macmillan Press Ltd.: London, UK, 1993, pp 54–80.

16. Petrusek, R. L.; Uhlenhopp, E. L.; Duteau, N.; Hurley, L. H. *J. Biol. Chem.* **1982**, *257*, 6207.
17. Thurston, D. E.; Bose, D. S. *Chem. Rev.* **1994**, *94*, 433.
18. Kamal, A.; Rao, M. V.; Laxman, N.; Ramesh, G.; Reddy, G. S. K. *Curr. Med. Chem. Anticancer Agents* **2002**, *2*, 215.
19. Gregson, S. J.; Howard, P. W.; Hartley, J. A.; Brooks, A. A.; Adams, L. J.; Jenkins, T. C.; Kelland, L. R.; Thurston, D. E. *J. Med. Chem.* **2001**, *44*, 737.
20. Kamal, A.; Ramesh, G.; Laxman, N.; Ramulu, P.; Srinivas, O.; Neelima, K.; Kondapi, A. K.; Srinu, V. B.; Nagarajaram, H. A. *J. Med. Chem.* **2002**, *45*, 4679.
21. Kamal, A.; Ramesh, G.; Srinivas, O.; Ramulu, P.; Laxman, N.; Rehana, T.; Deepak, M.; Achary, M. S.; Nagarajaram, H. A. *Bioorg. Med. Chem.* **2004**, *12*, 5427.
22. Kamal, A.; Ramulu, P.; Srinivas, O.; Ramesh, G.; Kumar, P. P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4791.
23. Shchekotikhin, A. E.; Shtil, A. A.; Luzikov, Y. N.; Bobrysheva, T. V.; Buyanov, V. N.; Preobrazhenskaya, M. N. *Biorg. Med.Chem.* **2005**, *13*, 2285.
24. Cholody, W. M.; Hernandez, L.; Hassner, L.; Scudiero, D. A.; Djurickovic, D. B.; Michejda, C. J. *J. Med. Chem.* **1995**, *38*, 3043.
25. Najajreh, Y.; Perez, J. M.; Navarro-Ranninger, C.; Gibson, D. *J. Med. Chem.* **2002**, *45*, 5189.
26. Kamal, A.; Laxman, N.; Ramesh, G.; Srinivas, O.; Ramulu, P. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1917.
27. Kamal, A.; Reddy, P. S. M. M.; Reddy, D. R.; Laxman, E. *Bioorg. Med. Chem.* **2006**, *14*, 385.
28. Kamal, A.; Ramulu, P.; Srinivas, O.; Ramesh, G. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3517.
29. Bose, D. S.; Thompson, A. S.; Ching, J.; Hartley, J. A.; Berardini, M. D.; Jenkins, T. C.; Neidle, S.; Hurley, L. H.; Thruston, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 4939.
30. Balcarova, Z.; Mrazek, J.; Kleinwachter, V.; Brabec, V. Gen. Physiol. *Biophysics* **1992**, *11*, 579.
31. Jones, G. B.; Davey, C. L.; Jenkins, T. C.; Kamal, A.; Kneale, G. G.; Neidle, S.; Webster, G. D.; Thurston, D. E. *Anti-Cancer Drug Des.* **1990**, *5*, 249.
32. Connaughie, A. W. M.; Jenkins, T. C. *J. Med. Chem.* **1995**, *38*, 3488.
33. alcarova, Z.; Mrazek, J.; Kleinwachter, V.; Brabec, V. Gen. Physiol. *Biophysics* **1992**, *11*, 579.
34. Brabec, V.; Balcarova, Z. *Eur. J. Biochem.* **1993**, *216*, 183.
35. Collier, D. A.; Thuong, N. T.; Helene, C. *J. Am. Chem. Soc.* **1991**, *113*, 1457.
36. Puvvada, M. S.; Hartley, J. A.; Jenkins, T. C.; Thurston, D. E. *Nucleic Acids Res.* **1993**, *21*, 3671.
37. Kamal, A.; Khan, M. N. A.; Srinivas, R. K.; Kaleem, A. S.; Shiva, K. M.; Juvekar, A.; Sen, S.; Zingde, S. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5400.
38. Gregson, S. J.; Howard, P. W.; Gullick, D. R.; Hamaguchi, A.; Corcoran, K. E.; Brooks, N. A.; Hartley, J. A.; Jenkins, T. C.; Patel, S.; Guille, M. J.; Thruston, D. E. *J. Med. Chem.* **2004**, *47*, 1161.
39. Sumner, W.; Bennett, G. N. *Nucleic Acids Res.* **1981**, *9*, 2105.
40. Vichai, V.; Kirtikara, K. *Nat. Protoc.* **2006**, *1*, 1112.
41. Skehn, P.; Storeng, R.; Scudiero, A.; Monks, J.; McMohan, D.; Vistica, D.; Jonathan, T. W.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.