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Remarkable DNA binding affinity and potential anticancer activity of pyrrolo[2,1-*c*][1,4]benzodiazepine–naphthalimide conjugates linked through piperazine side-armed alkane spacers

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ABSTRACT

A series of pyrrolobenzodiazepine–naphthalimide conjugates tethered through a piperazine ring system have been designed, synthesized, and evaluated for their anticancer activity. These new conjugates exhibit very high DNA binding affinity and cytotoxic activity against a number of cell lines.

1. Introduction

Efforts to increase the DNA recognizing ability of DNA interacting agents has led to the approach of dimerizing such molecules. In the past few years, a variety of dimeric DNA binders have been reported and some of them exhibited potent cytotoxicity and higher in vivo antitumor activity compared to the monomers.[1] Further, the bifunctional DNA-interactive agents in which the two functionalities possess two different mechanisms of interaction with DNA and are capable of recognizing heterogeneous DNA sequences, coupled by a spacer, have also attracted considerable attention as a new class of antitumor agents.[2] In this strategy, coupling of a groove binder with a DNA-affinic intercalator or coupling two groove-binding moieties provides a basis for modulating the sequence-selective binding behavior or tailoring the hybrid ligands for mixed-sequence recognition.

Naphthalimides are DNA-intercalating agents with high antitumor activity,[1a] which bind to DNA by insertion between the base pairs of the double helix. The main driving forces for their DNA binding ability are charge transfer interactions and stacking between the base pairs. The development of naphthalimide class of compounds as anticancer agents started in the year 1973. Both mitonafide (**1**) and amonafide (**2**), as shown in Figure 1, have been tested in clinical trials. Mitonafide has been studied in phase I and phase II clinical trials where it exhibited good activity against solid tumors.[3] Unfortunately, mitonafide was found to possess central nervous system toxicity. Two other compounds of the bis-naphthalimide series, LU 79553 (**3**) and DMP 840 (**4**), are reported to be in clinical trials.[4–6] The absence of nitro group in the chromophore of LU 79553 may be beneficial, since the nitro group on the monomeric compounds was expected to be the cause for the central nervous system toxicity as observed in the clinical trials of mitonafide.

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a group of antitumor agents which include natural products such as anthramycin, tomaymycin, sibiromycin, and DC-81 (**5**).[1c] They exert their biological activity by covalent binding to the minor groove of the DNA to form an aminal linkage between the electrophilic carbinolamine present at the C11 position and the N2 of the guanine.[7] Molecular modeling, solution NMR, fluorimetry, and DNA footprinting experiments reveal that the PBD monomers recognize three base pairs of DNA with an alkylating preference for Pu-G-Pu sequences. Recently, there has been increasing interest in the design and synthesis of DNA interstrand crosslinking agents as well as conjugates to enhance the sequence selectivity and increase selectivity for tumor cells.[6] One of the recently developed PBD dimers, SJG-136 (**6**) with C2-exomethylene substitution has

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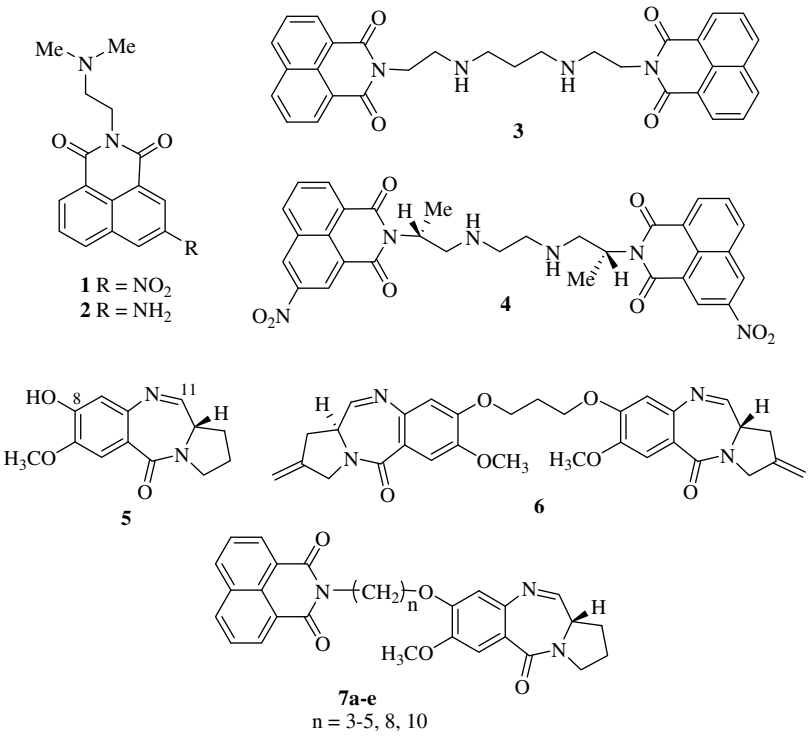


Figure 1. Chemical structures of mitonafide (**1**), amonafide (**2**), LU 79553 (**3**), DMP 840 (**4**), DC-81 (**5**), SJG-136 (**6**), and PBD–naphthalimide hybrids (**7a–e**).

been selected for clinical trials.[8] Cyclopropaindole–PBD,[9,10] pyrrole and imidazole polyamide–PBD,[11–13] and cyclic amine-PBD[14] conjugates have been designed and synthesized to explore their anticancer potential and DNA binding ability. The type of spacer employed to link the two pharmacophores plays an important role in the design of such hybrid molecules. An appropriately optimized flexible linker leads to improved cytotoxicity. In view of the interesting biological activity exhibited by PBDs, there has been considerable interest in the structural modification of PBDs in our laboratory.[15–17] In this endeavor a series of novel PBD–naphthalimide hybrids[18] (**7a–e**) have been prepared, which exhibited promising anticancer activity against numerous cell lines. The National Cancer Institute (NCI), USA has investigated the cytotoxicity of a member of this series, **7c**, using hollow fiber assay. This compound exhibited a good cell killing ability at a concentration of 37.5 mg/kg/dose in selected cell lines. In view of its promising activity it has been selected for detailed studies by the NCI. In continuation to the efforts in this direction, we now report the synthesis and biological evaluation of a new series of PBD–naphthalimide hybrids, coupled through a piperazine moiety with side-armed symmetrical and unsymmetrical alkane spacers.[19]

2. Chemistry

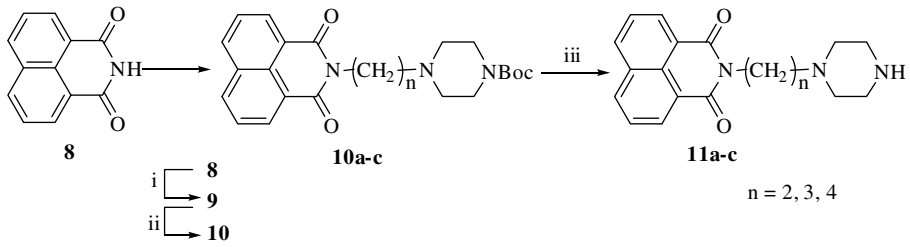
The synthesis of new PBD hybrids was carried out employing piperazine-linked naphthalimides **11a–c**. These intermediates were obtained by alkylation of naphthalimide with dibromoalkanes followed by coupling with *N*-Boc piperazine and deprotection of Boc with trifluoroacetic acid. The mono alkylation of **8** was achieved by using excess dibromoalkanes (Scheme 1).

Synthesis of the other precursor (2*S*)-*N*-[4-(ω-bromoalkyloxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethylthioacetals **13a–c** was carried out in six steps according to the literature method,[17] starting from the commercially available vanillin and coupling of 4-(ω-bromoalkyloxy)-5-methoxy-2-nitrobenzoic acid and (2*S*)-pyrrolidine-2-carboxaldehyde diethylthioacetal as the key step. The nitrothioacetals **13a–c** were coupled with 2-[ω-(piperazine-1-yl)alkyl]benz[*de*]isoquinoline-1,3-diones **(11a–c)** to give C8-linked naphthalimide nitrothioacetals **14a–e**. The nitrothioacetals **13b** and **13c**, having three and four carbon spacer, were coupled with piperazine-linked naphthalimide intermediate **11a**, having two carbon spacers, providing C8-linked naphthalimide–piperazine–nitrothioacetals **14b** and **14c,** consisting of unsymmetrical alkane spacers. The nitrothioacetals, **13a–c** were coupled with the corresponding piperazine-linked naphthalimide intermediates **11a–c** having identical alkane spacer to give C8-linked naphthalimide nitrothioacetals **14a, 14d** and **14e.** The nitro group of these compounds has been efficiently reduced employing $SnCl_2{\cdot}2H_2O$ to afford the corresponding aminothioacetals **15a–e**. Deprotection of the thioacetal groups with $HgCl_2$ and $CaCO_3$ afforded the desired naphthalimide–PBD hybrids **16a–e** (Scheme 2).

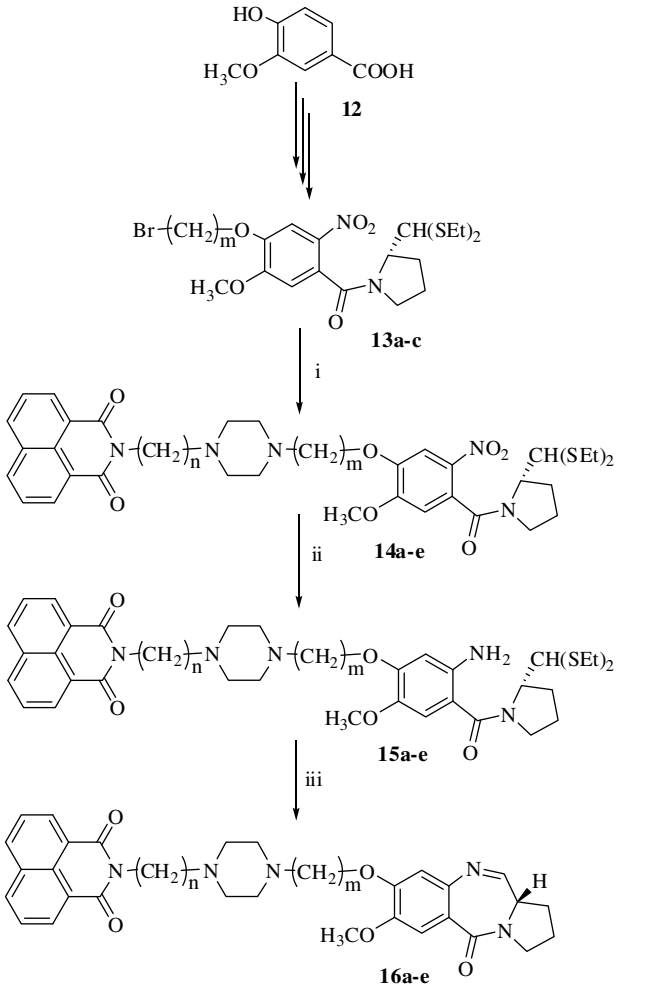
3. DNA interactions: thermal denaturation studies

The DNA binding affinity of these new PBD hybrids **16a–e** has been evaluated through thermal denaturation studies with duplex-form of calf thymus DNA (CT-DNA) by using modified reported procedure.[20,21] The thermal denaturation studies for these compounds have been carried out at PBD/DNA molar ratios of 1:5. The increase in melting temperature (ΔT_m) for each compound has been examined at 0 and 18 h of incubation at 37 °C (Table 1). Data for DC-81 and DSB-120 are included in Table 1 for comparison.

The results show that these hybrids stabilize the thermal helix → coil transition (T_m) for CT-DNA duplex more efficiently. There is a slight increase in melting temperature, when the incubation time is increased from 0 to 18 h at 37 °C. The new PBD–naphthalimide hybrids have shown ΔT_m values ranging from 12.9 to 26.5 °C after incubation for 18 h. In this series, compound **16d**, which has a three-carbon chain on either side of piperazine, has lower DNA binding affinity compared to other compounds, and this compound elevates the hybrid melting temperature by 12.9 °C after incubation for 18 h. Compound **16b,** which has piperazine side armed



Scheme 1. Reagents and conditions: (i) dibromo alkanes, K_2CO_3, acetonitrile, reflux, 12 h; (ii) *N*-Boc piperazine, K_2CO_3, acetonitrile, reflux, 8 h; (iii) CF_3COOH, $CHCl_3$, rt, 8 h.



Scheme 2. Reagents and conditions: (i) compounds **11a–c**, K_2CO_3, acetonitrile, reflux, 12 h; (ii) $SnCl_2$–$2H_2O$, methanol, reflux, 3.5 h; (iii) $HgCl_2$, $CaCO_3$, acetonitrile/H_2O, rt, 15 h.

with two- and three-carbon chains, has higher DNA binding affinity and elevates the helix melting temperature of CT-DNA by 26.5 °C after incubation at 37 °C for 18 h. The PBD hybrids **16c** and **16d**, which have six carbon alkane spacer ($m + n$), between PBD and naphthalimide, but differ in carbon spacers between PBD–piperazine and piperazine–naphthalimide moieties, have shown different DNA binding affinity. With the increase in the chain length of the linker connecting the naphthalimide to piperazine from two to three carbons, a decrease in melting temperature was observed, followed by an increase from three to four carbons. This illustrates the significant effect of alkane spacers between PBD–piperazine–naphthalimide moieties. The spacers in **16b** and **16c** appear to impart the molecules a better fit in the minor groove of DNA, leading to enhanced binding affinity. In the same experiment, the naturally occurring DC-81 exhibits a ΔT_m of 0.7 °C and its dimer (DSB-120) gives a ΔT_m of 15.4 °C. This demonstrates that these new PBD hybrids have remarkable DNA binding affinity. It is interesting to note that these hybrids with unsymmetrical side chains (**16b–c**) have higher ΔT_m values than those with identical side chains (**16a** and **16c** and **16d**).

Table 1
Thermal denaturation data for PBD–naphthalimide hybrids (**16a–e**) with calf thymus (CT) DNA

Compound[b]	Induced ΔT_m[a] (°C) after incubation at 37 °C	
	0 h	18 h
16a	21.9	22.7
16b	25.9	26.5
16c	23.1	23.9
16d	12.0	12.9
16e	19.9	20.8
DC-81	0.3	0.7
DSB-120	10.2	15.1

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.4 °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].

4. Cytotoxicity

Compounds **16a–c** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines such as Hop62 (lung), SiHa (cervix), MCF7 and ZR-75-1 (breast), Colo205 (colon), PC3 (prostate), and A2780 (ovarian), by employing sulforhodamine B (SRB) assay. The results described in Table 2 show that all the

Table 2
In vitro anticancer activity data for the representative PBD–naphthalimide hybrids (**16a–c**)

Compound	IC_{50}[a] (μM)						
	Hop62[b]	SiHa[c]	MCF7[d]	ZR-75-1[d]	PC3[e]	Colo205[f]	A2780[g]
16a	1.2	1.0	1.4	<1.0	0.8	1.6	0.5
16b	<1.0	0.7	0.5	<1.0	0.5	0.5	0.5
16c	<1.0	0.8	0.5	<1.0	0.5	0.5	0.6
ADR	1	1.5	0.5	0.6	0.5	2.0	0. 5

[a] Dose of the compound required to inhibit cell growth by 50% compared to untreated cell controls. Values are derived from IC_{50} graphs. All experiments were done in triplicate wells and each experiment was repeated thrice.
[b] Lung cancer.
[c] Cervix cancer.
[d] Breast cancer.
[e] Prostate cancer.
[f] Colon cancer.
[g] Ovarian cancer.

new compounds are significantly cytotoxic, with the concentration of the drug that produced 50% inhibition of cell growth (IC_{50}) ranging from 0.5 to 1.6 μM. Compounds **16b** and **16c**, which have shown higher DNA binding affinity, displayed higher cytotoxicity with IC_{50} values ranging between 0.5 and 1 μM against all the cell lines examined, when compared to **16a**, that comprised symmetrical alkane spacers exhibiting a correlation between ΔT_m and cytotoxicity of the molecules. Compound **16a** exhibited higher cytotoxicity against A2780, PC3, and SiHa cell lines with IC_{50} values ranging from 0.5 to 1.0 μM.

5. Conclusion

A series of new PBD conjugates, in which DNA covalent binding PBD moiety conjugated with a DNA-intercalating naphthalimide ring system, through piperazine moiety with carbon side chain arms have been designed, synthesized, and evaluated for their biological activity. These unique bifunctional compounds exhibit remarkable DNA binding ability in comparison to some of the cross linking agents. These compounds also exhibit potent in vitro antitumor activity against some of the cancer cell lines examined.

6. Experimental

6.1. 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). Most of the reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh: dichloromethane (calcium hydride), 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.

6.2. 2-(2-bromoethyl)benz[*de*]isoquinoline-1,3-dione (9a)

To a solution of compound **8** (197 mg, 1 mmol) in acetonitrile (30 mL), anhydrous potassium carbonate (553 mg, 4 mmol) and 1,2-dibromoethane (561 mg, 3 mmol) were added and the mixture was refluxed for 12 h. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography (10% EtOAc–hexane) to afford the compound **9a** (273 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 3.62 (t, 2H, J = 7.1 Hz, CH_2Br), 4.56 (t, 2H, J = 7.1 Hz, NCH_2), 7.74 (t, 2H, J = 7.6 Hz, ArH), 8.18 (d, 2H, J = 8.2 Hz, ArH), 8.58 (d, 2H, J = 7.2 Hz, ArH); MS (EI): m/z 304 $[M]^+$.

6.3. 2-(3-bromopropyl)benz[*de*]isoquinoline-1,3-dione (9b)

The compound **9b** was prepared following the method described for the preparation of the compound **9a**, employing **8** (197 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 **9b** (280 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 2.10–2.40 (m, 2H, CH_2), 3.40–3.58 (m, 2H, CH_2Br), 4.22–4.40 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.20 (d, 2H, J = 8.0 Hz, ArH), 8.60 (d, 2H, J = 7.4 Hz, ArH); MS (EI): m/z 318 $[M]^+$.

6.4. 2-(4-bromobutyl)benz[*de*]isoquinoline-1,3-dione (9c)

The compound **9c** was prepared following the method described for the preparation of the compound **9a**, employing **8** (197 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 **9c** (305 mg, 92%). ^{1}H NMR ($CDCl_3$): δ 1.80–2.15 (m, 4H, 2 × CH_2), 3.38–3.52 (m, 2H, CH_2Br), 4.05–4.22 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.2 Hz, ArH), 8.20 (d, 2H, J = 8.0 Hz, ArH), 8.60 (d, 2H, J = 7.4 Hz, ArH); MS (EI): m/z 332 $[M]^+$.

6.5. 2-[2′-[4-(*tert*-Butoxycarbonyl)piperazine-1-yl]ethyl]benz[*de*]isoquinoline-1,3-dione (10a)

To a solution of 2-(2-bromoethyl)benz[*de*]isoquinoline-1,3-dione (**9a**) (304 mg, 1 mmol) in dry acetone (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the *N*-Boc piperazine (186 mg, 1 mmol). The reaction mixture was refluxed for 8 h. The reaction was monitored by TLC using ethyl acetate/hexane (4:1) as a solvent system. The potassium carbonate was removed by suction filtration, the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **10a** (348 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.42 (s, 9H, $C(CH_3)_3$), 2.52 (t, 4H, J = 4.9 Hz, 2 × NCH_2), 2.68 (t, 2H, J = 6.3 Hz, NCH_2), 3.35 (t, 4H, J = 4.6 Hz, 2 × NCH_2), 4.29 (t, 2H, J = 6.1 Hz, NCH_2), 7.73 (t, 2H, J = 7.4 Hz, ArH), 8.12 (d, 2H, J = 8.4 Hz, ArH), 8.55 (d, 2H, J = 7.2 Hz, ArH); MS (FAB): 410 $[M+1]^+$.

6.6. 2-[3′-[4-(*tert*-Butoxycarbonyl)piperazine-1-yl]propyl]benz[*de*]isoquinoline-1,3-dione (10b)

The compound **10b** was prepared following the method described for the preparation of the compound **10a,** employing *N*-Boc piperazine and **9b** (318 mg, 1 mmol), and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **10b** (364 mg, 86%). ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9H, $C(CH_3)_3$), 1.82–2.0 (m, 2H, CH_2), 2.40–2.60 (m, 6H, 3 × NCH_2), 3.22–3.40 (m, 4H, 2 × NCH_2), 4.10–4.25 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.18 (d, 2H, J = 8.0 Hz, ArH), 8.55 (d, 2H, J = 7.5 Hz, ArH); MS (FAB): 424 $[M+1]^+$.

6.7. 2-[4′-[4-(*tert*-Butoxycarbonyl)piperazine-1-yl]butyl]benz[*de*]isoquinoline-1,3-dione (10c)

The compound **10c** was prepared following the method described for the preparation of the compound **10a,** employing *N*-Boc piperazine and **9c** (332 mg, 1 mmol), and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **10c** (372 mg, 85%). ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9H, $C(CH_3)_3$), 1.60–1.80 (m, 4H, 2 × CH_2), 2.50–2.72 (m, 6H, 3 × NCH_2), 3.20–3.35 (m, 4H, 2 × NCH_2), 4.10–4.24 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.20 (d, 2H, J = 8.2 Hz, ArH), 8.60 (d, 2H, J = 7.6 Hz, ArH); MS (FAB): 437 $[M]^+$.

6.8. 2-[2′-(Piperazine-1-yl)ethyl]benz[*de*]isoquinoline-1,3-dione (11a)

To a solution of **10a** (409 mg, 1 mmol) in chloroform (15 mL) was added trifluoroacetic acid (1.14 g, 10 mmol) and the mixture

was stirred at room temperature for 12 h. The solution was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with chloroform (2× 15 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to afford the compound **11a** (238 mg, 77%). ^{1}H NMR ($CDCl_3$): δ 2.45–2.65 (m, 6H, 3 × NCH_2), 2.80–2.92 (m, 4H, 2 × NCH_2), 4.20–4.35 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.20 (d, 2H, J = 8.2 Hz, ArH), 8.60 (d, 2H, J = 7.6 Hz, ArH); MS (EI): m/z 310 $[M+1]^+$.

6.9. 2-[3′-(Piperazine-1-yl)propyl]benz[*de*]isoquinoline-1,3-dione (11b)

The compound **11b** was prepared following the method described for the preparation of the compound **11a,** employing the compound **10b** (424 mg, 1 mmol) to afford the compound **11b** (246 mg, 76%). ^{1}H NMR ($CDCl_3$): δ 1.80–2.0 (m, 2H, CH_2), 2.25–2.40 (m, 6H, 3 × NCH_2), 2.85–3.0 (m, 4H, 2 × NCH_2), 4.15–4.30 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.4 Hz, ArH), 8.20 (d, 2H, J = 8.0 Hz, ArH), 8.60 (d, 2H, J = 7.5 Hz, ArH); MS (EI): m/z 324 $[M+1]^+$.

6.10. 2-[4′-(Piperazine-1-yl)butyl]benz[*de*]isoquinoline-1,3-dione (11c)

The compound **11c** was prepared following the method described for the preparation of the compound **11a,** employing the compound **10c** (438 mg, 1 mmol) to afford the compound **11c** (263 mg, 78%). ^{1}H NMR ($CDCl_3$): δ 1.50–1.80 (m, 4H, 2 × CH_2), 2.22–2.45 (m, 6H, 3 × NCH_2), 2.80–2.96 (m, 4H, 2 × NCH_2), 4.05–4.20 (m, 2H, NCH_2), 7.75 (t, 2H, J = 7.4 Hz, ArH), 8.20 (d, 2H, J = 8.0 Hz, ArH), 8.60 (d, 2H, J = 7.6 Hz, ArH); MS (EI): m/z 338 $[M+1]^+$.

6.11. (2*S*)-*N*-{4-[2-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-ethyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (14a)

To a solution of (2*S*)-*N*-[4-(2-bromoethoxy)-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethylthioacetal (**13a**) (507 mg, 1 mmol) in dry acetonitrile (30 mL) were added anhydrous K_2CO_3 (552 mg, 4 mmol) and the compound **11a** (309 mg, 1 mmol). The reaction mixture was refluxed for 12 h. The reaction was monitored by TLC using methanol/ethylacetate (1:19) as a solvent system. The potassium carbonate was removed by suction filtration, the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (5% MeOH–EtOAc) to afford the compound **14a** (588 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.22–1.40 (m, 6H, 2 × SCH_2CH_3), 1.70–2.35 (m, 4H, 2 × CH_2), 2.55–2.95 (m, 16H, 6 × NCH_2, 2 × SCH_2), 3.15–3.32 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 4.15–4.35 (m, 4H, OCH_2, NCH_2), 4.57–4.72 (m, 1H, CH), 4.80 (d, 1H, J = 4.3 Hz, $CH(SEt)_2$), 6.77 (s, 1H, ArH), 7.60–7.80 (m, 3H, ArH), 8.20 (t, 2H, J = 8.0 Hz, ArH), 8.55 (d, 2H, J = 7.6 Hz, ArH); FABMS: 736 $[M]^+$.

6.12. (2*S*)-*N*-{4-[3-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-propyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (14b)

The compound **14b** was prepared following the method described for the compound **14a,** employing **13b** (521 mg, 1 mmol) and **11a** (309 mg, 1 mmol), and the crude product was purified by column chromatography (5% MeOH–EtOAc) to afford the compound **14b** (615 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 1.30–1.42 (m, 6H, 2 × SCH_2CH_3), 1.70–2.30 (m, 6H, 3 × CH_2), 2.40–2.82 (m, 16H, 6 × NCH_2, 2 × SCH_2), 3.15–3.30 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 4.10–4.20 (m, 2H, OCH_2), 4.26–4.36 (m, 2H, NCH_2), 4.60–4.70 (m, 1H, CH), 4.82 (d, 1H, J = 4.3 Hz, $CH(SEt)_2$), 6.77 (s, 1H, ArH), 7.65 (s, 1H, ArH), 7.75 (t, 2H, J = 7.4 Hz, ArH), 8.20 (d, 2H, J = 8.0 Hz, ArH), 8.6 (d, 2H, J = 7.6 Hz, ArH); FABMS: 750 $[M]^+$.

6.13. (2*S*)-*N*-{4-[4-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-butyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (14c)

The compound **14c** was prepared following the method described for the compound **14a,** employing **13c** (535 mg, 1 mmol) and **11a** (309 mg, 1 mmol), and the crude product was purified by column chromatography (5% MeOH–EtOAc) to afford the compound **14c** (611 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.40 (m, 6H, 2 × SCH_2CH_3), 1.60–2.15 (m, 8H, 4 × CH_2), 2.35–2.85 (m, 16H, 6 × NCH_2, 2 × SCH_2), 3.15–3.30 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 4.10–4.15 (m, 2H, OCH_2), 4.22–4.36 (m, 2H, NCH_2), 4.60–4.72 (m, 1H, CH), 4.80 (d, 1H, J = 4.2 Hz, $CH(SEt)_2$), 6.75 (s, 1H, ArH), 7.60 (s, 1H, ArH), 7.75 (t, 2H, J = 7.4 Hz, ArH), 8.20 (d, 2H, J = 8.2 Hz, ArH), 8.56 (d, 2H, J = 7.6 Hz, ArH); MS (FAB): 765 $[M+1]^+$.

6.14. (2*S*)-*N*-{4-[3-[4-[3-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)propyl]piperazin-1-yl]propyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (14d)

The compound **14d** was prepared following the method described for the compound **14a,** employing **13b** (521 mg, 1 mmol) and **11b** (323 mg, 1 mmol), and the crude product was purified by column chromatography (5% MeOH–EtOAc) to afford the compound **14d** (626 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.42 (m, 6H, 2 × SCH_2CH_3), 1.70–2.40 (m, 8H, 4 × CH_2), 2.60–3.30 (m, 18H, 6 × NCH_2, 2 × SCH_2, CH_2), 3.92 (s, 3H, OCH_3), 4.05–4.30 (m, 4H, OCH_2, NCH_2), 4.70–4.80 (m, 1H, CH), 4.82 (d, 1H, J = 4.2 Hz, $CH(SEt)_2$), 6.77 (s, 1H, ArH), 7.60 (s, 1H, ArH), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.18 (d, 2H, J = 8.0 Hz, ArH), 8.55 (d, 2H, J = 7.5 Hz, ArH); MS (FAB): 764 $[M]^+$.

6.15. (2*S*)-*N*-{4-[4-[4-[4-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)butyl]piperazin-1-yl]-butyl]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (14e)

The compound **17e** was prepared following the method described for the compound **14a,** employing **13c** (535 mg, 1 mmol) and **11c** (337 mg, 1 mmol), and the crude product was purified by column chromatography (5% MeOH–EtOAc) to afford the compound **14e** (641 mg, 81%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.40 (m, 6H, 2 × SCH_2CH_3), 1.60–2.33 (m, 12H, 6 × CH_2), 2.52–3.0 (m, 16H, 6 × NCH_2, 2 × SCH_2), 3.12–3.30 (m, 2H, CH_2), 3.95 (s, 3H, OCH_3), 4.02–4.25 (m, 4H, OCH_2, NCH_2), 4.60–4.72 (m, 1H, CH), 4.80 (d, 1H, J = 4.3 Hz, $CH(SEt)_2$), 6.75 (s, 1H, ArH), 7.60 (s, 1H, ArH), 7.75 (t, 2H, J = 7.4 Hz, ArH), 8.18 (d, 2H, J = 8.2 Hz, ArH), 8.56 (d, 2H, J = 7.6 Hz, ArH); MS (FAB): 793 $[M+1]^+$.

6.16. (2*S*)-*N*-{4-[2-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-ethyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (15a)

The compound **14a** (736 mg, 1 mmol) was dissolved in methanol (20 mL) and $SnCl_2{\cdot}2H_2O$ (1.13 g, 5 mmol) was added and the mixture was refluxed for 3.5 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 reduced pressure to afford the crude amino diethyl thioacetal **15a** as yellow oil (536 mg, 76%), which due to potential stability problems was directly used in the next step without isolation.

6.17. (2*S*)-*N*-{4-[3-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-propyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (15b)

The compound **15b** was prepared following the method described for the compound **15a**, employing the compound **14b** (750 mg, 1 mmol) to afford the amino diethyl thioacetal **15b** as a yellow oil (561 mg, 78%).

6.18. (2*S*)-*N*-{4-[4-[4-[2-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]-butyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (15c)

The compound **15c** was prepared following the method described for the compound **15a**, employing the compound **14c** (764 mg, 1 mmol) to afford the amino diethyl thioacetal **15c** as a yellow oil (536 mg, 73%).

6.19. (2*S*)-*N*-{4-[3-[4-[3-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)propyl]piperazin-1-yl]-propyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (15d)

The compound **15d** was prepared following the method described for the compound **15a**, employing the compound **14d** (764 mg, 1 mmol) to afford the amino diethyl thioacetal **15d** as a yellow oil (557 mg, 76%).

6.20. (2*S*)-*N*-{4-[4-[4-[4-(1,3-Dioxo-benz[*de*]isoquinolin-2-yl)butyl]piperazin-1-yl]-butyl]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (15e)

The compound **15e** was prepared following the method described for the compound **15a**, employing the compound **14e** (792 mg, 1 mmol) to afford the amino diethyl thioacetal **15e** as a yellow oil (618 mg, 78%).

6.21. 7-Methoxy-8-{2-[4-[2-(1,3-dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]ethyl}-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazep-in-5-one (16a)

A solution of amino thioacetal **15a** (706 mg, 1 mmol), $HgCl_2$ (597 mg, 2.2 mmol), and $CaCO_3$ (240 mg, 2.4 mmol) in acetonitrile-water (4:1) was stirred slowly at room temperature for 15 h. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through Celite. The clear yellow organic supernatant was extracted with ethyl acetate (2× 20 mL). The organic layer was washed with saturated $NaHCO_3$ (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure, and the crude product was purified by column chromatography (15% $CHCl_3$–MeOH) to afford the compound **16a** as a pale yellow solid (314 mg, 54%). This material was repeatedly evaporated from $CHCl_3$ in vacuum to generate the imine form. Mp 75–77 °C; $[\alpha]_D^2$6 +278.5 (*c* 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.90–2.40 (m, 4H, H-1, H-2), 2.45–2.92 (m, 12H, 6 × NCH_2), 3.55–3.82 (m, 3H, H-3, H-11a), 3.92 (s, 3H, OCH_3), 4.05–4.40 (m, 4H, OCH_2, NCH_2), 6.77 (s, 1H, H-6), 7.45 (s, 1H, H-9), 7.62 (d, 1H, *J* = 4.4 Hz, H-11), 7.76 (t, 2H, *J* = 7.7 Hz, ArH), 8.20 (d, 2H, *J* = 8.2 Hz, ArH), 8.60 (d, 2H, *J* = 7.3 Hz, ArH); FABMS: 582 $[M]^+$. Anal. Calcd for $C_{33}H_{35}N_5O_5$: C, 68.14; H, 6.07; N, 12.04. Found: C, 68.10; H, 6.11; N, 12.01.

6.22. 7-Methoxy-8-{3-[4-[2-(1,3-dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl] propyl}-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo[2,1-*c*][1,4]benzodiaze-pin-5-one (16b)

The compound **16b** was prepared following the method described for the preparation of the compound **16a**, employing **15b** (720 mg, 1 mmol) to afford the compound **16b** as a pale yellow solid (304 mg, 51%). Mp 81–83 °C; $[\alpha]_D^2$6 +282.5 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.60–2.16 (m, 6H, H-1, H-2, side-chain CH_2), 2.25–2.80 (m, 12H, 6 × NCH_2), 3.50–3.82 (m, 3H, H-3, H-11a), 3.94 (s, 3H, OCH_3), 4.05–4.18 (m, 2H, OCH_2), 4.22–4.38 (m, 2H, NCH_2), 6.80 (s, 1H, H-6), 7.45 (s, 1H, H-9), 7.62 (d, 1H, *J* = 4.3 Hz, H-11), 7.76 (t, 2H, *J* = 7.3 Hz, ArH), 8.20 (d, 2H, *J* = 8.0 Hz, ArH), 8.60 (d, 2H, *J* = 7.3 Hz, ArH); MS (FAB): 597 $[M+1]^+$. Anal. Calcd for $C_{34}H_{37}N_5O_5$: C, 68.55; H, 6.26; N, 11.76. Found: C, 68.58; H, 6.24; N, 11.80.

6.23. 7-Methoxy-8-{4-[4-[2-(1,3-dioxo-benz[*de*]isoquinolin-2-yl)ethyl]piperazin-1-yl]butyl}-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiaze-pin-5-one (16c)

The compound **16c** was prepared following the method described for the preparation of the compound **16a**, employing **15c** (734 mg, 1 mmol) to afford the compound **16c** as a pale yellow solid (329 mg, 54%). Mp 72–74 °C; $[\alpha]_D^2$6 +286.5 (*c* 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.56–2.15 (m, 8H, H-1, H-2, 2 × side-chain CH_2), 2.25–2.80 (m, 12H, 6 × NCH_2), 3.45–3.82 (m, 3H, H-3, H-11a), 3.92 (s, 3H, OCH_3), 4.0–4.15 (m, 2H, OCH_2), 4.22–4.37 (m, 2H, NCH_2), 6.72 (s, 1H, H-6), 7.42 (s, 1H, H-9), 7.60 (d, 1H, *J* = 4.3 Hz, H-11), 7.72 (t, 2H, *J* = 7.4 Hz, ArH), 8.16 (d, 2H, *J* = 8.1 Hz, ArH), 8.56 (d, 2H, *J* = 7.4 Hz, ArH); MS (FAB): 611 $[M+1]^+$. Anal. Calcd for $C_{35}H_{39}N_5O_5$: C, 68.95; H, 6.45; N, 11.49. Found: C, 68.91; H, 6.50; N, 11.52.

6.24. 7-Methoxy-8-{3-[4-[3-(1,3-dioxo-benz[*de*]isoquinolin-2-yl)propyl]piperazin-1-yl]propyl}-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiaze-pin-5-one (16d)

The compound **16d** was prepared following the method described for the preparation of the compound **16a**, employing **15d** (734 mg, 1 mmol) to afford the compound **16d** as a pale yellow solid (323 mg, 53%). Mp 65–67 °C; $[\alpha]_D^2$6 +34.5 (*c* 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.75–2.18 (m, 8H, H-1, H-2, 2 × side-chain CH_2), 2.22–2.80 (m, 12H, 6 × NCH_2), 3.45–4.30 (m, 10H, H-3, H-11a, OCH_2, NCH_2, OCH_3), 6.75 (s, 1H, H-6), 7.45 (s, 1H, H-9), 7.60 (d, 1H, *J* = 4.2 Hz, H-11), 7.72 (t, 2H, *J* = 7.4 Hz, ArH), 8.20 (d, 2H, *J* = 8.1 Hz, ArH), 8.58 (d, 2H, *J* = 7.3 Hz, ArH); MS (FAB): 610 $[M]^+$. Anal. Calcd for $C_{35}H_{39}N_5O_5$: C, 68.95; H, 6.45; N, 11.49. Found: C, 68.92; H, 6.42; N, 11.54.

6.25. 7-Methoxy-8-{4-[4-[4-(1,3-dioxo-benz[*de*]isoquinolin-2-yl)butyl]piperazin-1-yl]butyl}-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiaze-pin-5-one (16e)

The compound **16e** was prepared following the method described for the preparation of the compound **16a**, employing **15e** (762 mg, 1 mmol) to afford the compound **16e** as a pale yellow solid (325 mg, 51%). Mp 68–70 °C; $[\alpha]_D^2$6 +58.4 (*c* 0.5, $CHCl_3$); ^{1}H NMR

($CDCl_3$): δ 1.50–2.10 (m, 12H, H-1, H-2, 4 × side-chain CH_2), 2.25–2.80 (m, 12H, 6 × NCH_2), 3.45–3.86 (m, 3H, H-3, H-11a), 3.95 (s, 3H, OCH_3), 4.05–4.25 (m, 4H, OCH_2, NCH_2), 6.75 (s, 1H, H-6), 7.45 (s, 1H, H-9), 7.62 (d, 1H, J = 4.2 Hz, , H-11), 7.75 (t, 2H, J = 7.3 Hz, ArH), 8.20 (d, 2H, J = 8.1 Hz, ArH), 8.56 (d, 2H, J = 7.4 Hz, ArH); MS (FAB): 638 $[M]^+$. Anal. Calcd for $C_{37}H_{43}N_5O_5$: C, 69.68; H, 6.80; N, 10.98. Found: C, 69.71; H, 6.76; N, 11.03.

6.26. DNA thermal denaturation studies

The DNA binding affinity of these novel PBD hybrids (**16a–e**) has been evaluated through thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using modified reported procedure.[20,21] Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM Na_2EDTA, pH 7.00 ± 0.01) containing CT-DNA (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in DMSO to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions 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 $\Delta T_m = T_m(\text{DNA + PBD}) - T_m$ (DNA alone), where the T_m value for the PBD-free CT-DNA is 69.4 °C ± 0.01.

6.27. In vitro evaluation of cytotoxic activity

The compounds **16a–c** were evaluated for in vitro activity against selected human tumor cell lines, derived from six cancer types (lung cancer, cervix cancer, breast cancer, prostate cancer, colon cancer, and ovarian cancer). For each compound, dose–response curves against each cell line were measured. Sulforhodamine B (SRB) protein assay[22,23] has been used to estimate cell viability or growth.

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