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Synthesis and cytotoxic activity of a new potential DNA bisintercalator: 1,4-Bis{3-[*N*-(4-chlorobenzo[*g*]phthalazin-1-yl)aminopropyl]}piperazine

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ABSTRACT

The synthesis of new 1,4-bisalkylamino (**2**–**4**) and 1-alkylamino-4-chloro (**5**–**6**) substituted benzo[g]phthalazines is reported. Compounds **2**–**4** and **6** were prepared both in the free and heteroaromatic ring protonated forms. Bifunctional **6** contains the 1,4-bisaminopropylpiperazine chain as a linker between the two heteroaromatic units, whereas **5** is its monofunctional analogue. The in vitro antitumour activity of the synthesized compounds has been tested against human colon, breast and lung carcinoma cells, and also against human glioblastoma cells. Results obtained show that all of them are active in all cases, but bifunctional **6**·2HCl is remarkably effective against the four cell lines tested, exhibiting IC_{50} values in the range of 10^{-7} M, similar to those found for doxorubicin. The bifunctional structure of **6**·2HCl enhances activity with respect to the monofunctional related compounds **5** and **7**, leading to the highest activity among all the compounds tested. Molecular modelling of **6** suggests that those results could be indicative of DNA bisintercalation, which should be specially favoured in the diprotonated form **6**·2HCl, a compound suitable for being studied more in depth in further biological tests. Measure of the DNA thermal melting curves show that the linear rise in T_m for bifunctional **6**·2HCl is nearly twice than that one obtained for monofunctional **5**, and supports the DNA-binding hypothesis.

1. Introduction

DNA as a carrier of genetic information is a major target for drug interaction due to the ability of DNA-bound drugs to interfere with transcription and/or replication, an essential step in cell growth and division. Nucleic acids interact reversibly with a broad range of chemical species that include water, metal ions and their complexes, small organic molecules, and proteins. Intercalating agents constitute the most important group of small organic molecules that interact reversibly with the DNA double helix. Some of them (i.e., daunorubicin, doxorubicin, idarubicin or mitoxantrone) are valuable drugs currently used for the treatment of ovarian and breast cancers and acute leukaemias.[1] Major features for achieving remarkable therapeutical results are: (i) an usually tricyclic planar chromophore able to insert into the base pairs of the nucleotides and (ii) a positive charge favouring electrostatic interactions with nucleotides.[2] That charge may be located in flexible side chains, as occurs in mitoxantrone,[3] or in an heteroaromatic ring containing easily protonable sp^2 nitrogens, as it is the case of amsacrine.[4] Furthermore, the simultaneous presence of two aromatic systems connected by an appropriate linker is considered to enhance the antitumour activity, since bisintercalation is usually associated with higher DNA-binding affinities and slower dissociation rates than those observed for the corresponding monomers.[5,6]

As a consequence of the above mentioned points, bifunctional heteroaromatic molecules with the two chromophores linked by flexible heteroaliphatic chains can be considered as targets in the design of new drugs with antitumour properties. Therefore, a high number of bifunctional structures derived form acridines,[7] phenazines,[8] indolocarbazoles,[9] naphthalimides,[10] anthracenyl isoxazolyl amides[11] or indenoisoquinolines,[12] are under study, and many of them have shown powerful cytotoxic activities.

In connection with this matter, our research group has been working in the preparation of two related series of nitrogenated heterocyclic compounds with the general structures A and B schematized in Figure 1. 1,4-Bisalkylaminobenzo[*g*]phthalazine derivatives of the type A were synthesized by heating 1,4-dichlorobenzo[*g*]phthalazine in the presence of an excess of the required amine in an autoclave without any solvent.[13] One of those compounds (Fig. 1A, X = OMe) was isolated as the monohydrochloride form, and its reaction with cobalt(II) dichloride in neutral

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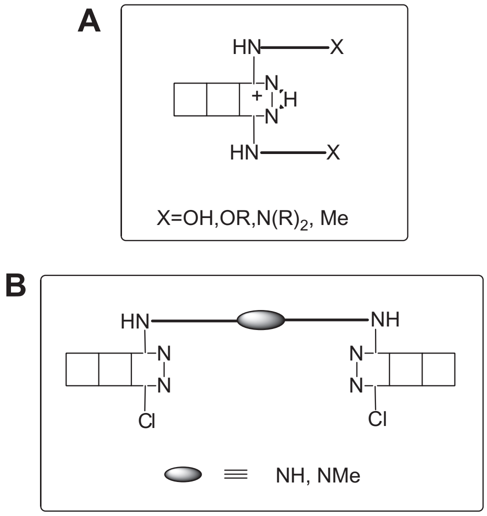


Figure 1.

medium afforded di-{1,4-bis-(3-methoxypropylamino)-3(2)*H*-benzo[*g*]phthalazinium}$_2$Co(II)Cl$_4$. The crystalline structure of this salt together with relevant spectroscopic data obtained from the starting monohydrochloride salt, demonstrated that protonation of the 1,4-bis(alkylamino)benzo-[*g*]phthalazine nucleus occurs at the ring nitrogen atoms, being the positive charge shared by the endo- and exocyclic nitrogen atoms.[14]

It was also verified that this tricyclic heteroaromatic nucleus may be protonated at physiological pH depending on the nature of the side chains. Thus, the pK_a values of 1,4-bis(propylamino)benzo-[*g*]phthalazines functionalized with cationic groups (Fig. 1A, X = NMe$_2$) are close to 6, while functionalization with neutral groups (Fig. 1A, X = OMe, OH) gives pK_a values higher than 8. By taking the 3-methoxypropyl derivative as a model, it was shown that the ionizable 1,4-bis(alkylamino)-benzo[*g*]phthalazine system interacts with DNA by intercalation at physiological pH. Intercalation induces a positive superhelical torsion effect on circular DNA related to that one found for ethidium bromide.[15]

On the other hand, we have found that derivatives of the type A in which the heteroatoms located at the end of the side chains are replaced by carbon atoms exhibit both cytotoxic and trypanosomicide activities.[16] Furthermore, the action of 1,4-bis(butylamino)benzo[*g*]phthalazine (ABP) on the cell cycle and differentiation of U-937 human promonocytic leukaemia cells has been studied and compared with that one of amsacrine (mAMSA).[17] At subcytotoxic concentrations, *m*AMSA and ABP reduced the proliferation activity to a similar extension and caused little mortality. The *m*AMSA induced the cell to accumulate at the G$_2$ phase of the cycle, while cycle inhibition originated by ABP was not phase specific. In addition, *m*AMSA caused an increase in the cell mass while ABP provoked cell shrinkage. This was consistent with the fact that ABP considerably inhibited protein synthesis, while *m*AMSA did not significantly affect this activity. Finally, both drugs inhibited RNA synthesis, but the inhibition was more prominent in the case of ABP.

In our previous work about the reactivity of 1,4-diclorobenzo[*g*]phthalazine with nucleophiles, we had found that the use of solvents of different nature allows handling the type of substitution at the pyridazine ring: acetonitrile only provides the 1-alkylamino-4-chlorosubstituted mono- and bifunctional products. This finding allowed us to prepare also bifunctional derivatives of the type B (Fig. 1), in which the two 1-aminopropylen-4-chlorobenzo[g]phthalazine fragments are linked by NH or NMe groups. Those compounds have shown in vitro cytotoxic activity against HT-29 human cells, that is enhanced by methyl substitution at the nitrogen atom.[18]

Finally, it has been reported that the introduction of a piperazine unit in the flexible linker of some bifunctional heteroaromatic molecules seems to favour biological activity. That is the case of imidazoacridones linked to a nitronaphthalimide moiety by a 1,4-dipropanepiperazine unit, that are selective and potent compounds against colon cancer and leukaemia,[19] and also of other symmetric bifunctional compounds with two heteroaromatic units connected by the same linker[20] that have shown to be effective against HIV-1 infections.

Taking into account all these previous findings, in this work we want to report the synthesis and in vitro antitumour activity data of two new monofunctional compounds with the schematic structure A: **3** and **4**, and of the related mono- and bifunctional compounds **5** and **6**, containing one or two benzo[*g*]phthalazine units linked to a 1,4-dipropanepiperazine chain (Fig. 2). Molecular modelling studies and DNA thermal melting experiments are also included in order to get some information regarding the potential intercalating features of the most active compound **6**, isolated as the double hydrochloride salt.

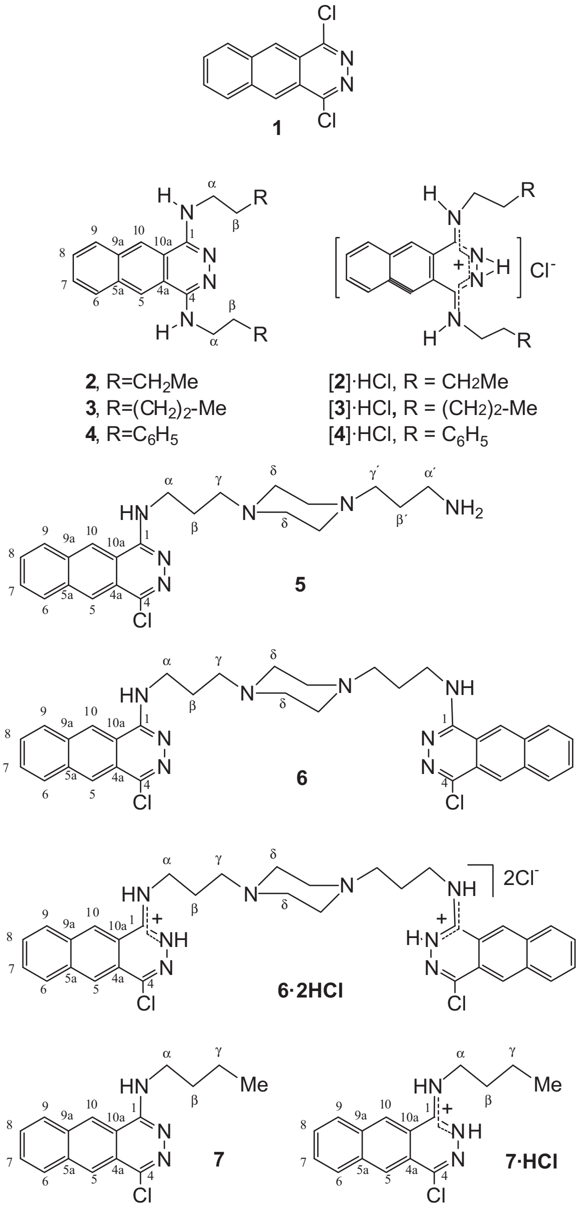


Figure 2.

2. Results and discussion

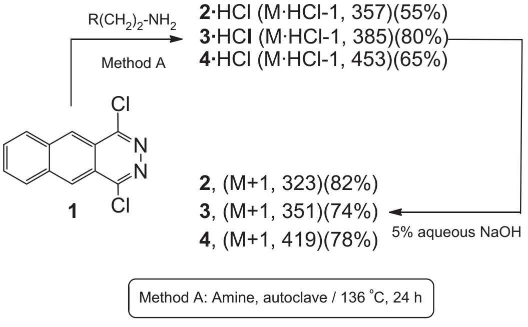
2.1. Chemistry

The preparation of compounds **2**–**4** in their hydrochloride form has been performed by nucleophilic substitution of 1,4-dichlorobenzo[*g*]-phthalazine according to a procedure previously patented by us.[13] The starting compound **1** was reacted in an autoclave with a great excess of butylamine, pentylamine or 2-phenylethylamine under conditions described in Scheme 1 (method A). In this way, the corresponding amine acts simultaneously as solvent, reactant, and acceptor of the hydrogen chloride excess. The hydrochlorides were obtained as yellow solids in yields ranging from 55% to 80%. Further treatment with aqueous NaOH led to the free compounds **2**–**4**, which were purified by column chromatography and isolated in the solid state with yields from 74% to 82%. The free compounds resulted to be very hygroscopic, and required filtration through basic Al_2O_3 in order to obtain them in anhydrous form.

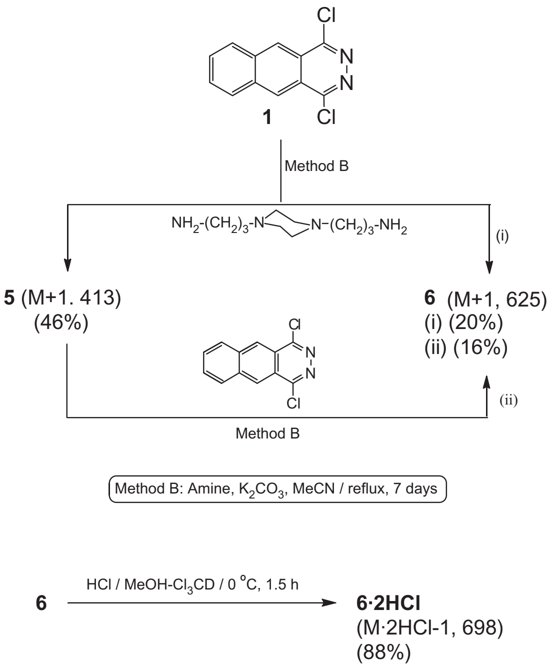
The monoalkylamino substituted compounds **5** and **6** were obtained by refluxing a 1:1 mixture of **1** and 1,4-bis(aminopropyl)piperazine in acetonitrile, in the presence of potassium carbonate (method B in Scheme 2). Chromatographic work up of the reaction crude in the presence of ammonium hydroxide allowed the isolation of **5** and **6** as the free compounds with 46% and 20% yields, respectively. When mononuclear compound **5** was reacted with **1** under the same conditions (method B), a supplementary amount of **6** could be obtained in a 16% yield. On the other hand, **6** was exclusively formed as the double hydrochloride salt by treating free **6** with HCl generated in situ in a MeOH/Cl_3CH solution and further insolubilization of the hydrochloride by ethyl ether addition.

All the new compounds synthesized were unequivocally identified by their elemental microanalysis, IR, ^1H NMR, and ^{13}C NMR spectroscopic data, as shown in Section 3. Mass spectra of the free compounds were recorded as ESI in positive (M+1) mode, and those of the hydrochlorides in negative (M·HCl−1) mode. Schemes 1 and 2 display the molecular ions obtained from the mass spectra of all of them, which agree in all cases with the proposed structures.

For an accurate assignment of the ^1H and ^{13}C NMR spectra signals, gHMBC and gHMQC experiments were especially useful. The mono- and dialkylamino substitution products are easily differentiated in both ^1H and ^{13}C NMR spectra on the basis of signals corresponding to rings A and B of the polyheterocyclic moiety. Protons H_5 and H_{10}, which appear as an unique singlet in the ^1H NMR spectra of **2**–**4**, lose their equivalence in the monosubstituted compounds **5** and **6**, with their chemical shifts differing in 0.14 and 0.21 ppm, respectively (Table 1). Accordingly, the carbon atom attached to the alkylamino substituent (C_1) is always deshielded with respect to that one neighbouring the chlorine atom in the ^{13}C NMR spectra: carbons C_1 and C_4, identical in **2**–**4**, show variations of 14.3 and 9.64 ppm in, respectively, **5** and **6**. The carbon pairs 4a/10a and 5/10 follow also the same pattern, although chemical shifts differences are substantially lower. In a similar way, carbons and protons of the two aliphatic chains exhibit a symmetric pattern in **6**, but are differentiated as two distinct propane units in **5**.



Scheme 1.

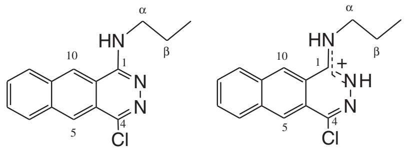


Scheme 2.

To establish the protonation sites of **6**, a comparison between the NMR spectra of free **6** and of **6**·2HCl (Table 1) is relevant. The spectra of both compounds, registered in DMSO-d_6, show that H_{10}, neighbouring the alkylamino group, is deshielded by 0.78 ppm in the hydrochloride salt, whereas H_5 is only shifted by 0.20 ppm. Furthermore, the hydrogen atoms in α respect to the same group experiment a shielding of 0.27 ppm in the protonated form. Those facts point out towards protonation of the amino-imino moiety, and not of the isolated piperazine ring. The protons of the ammonium group appear in the spectrum of the hydrochloride salt as a broad signal centred at 10.85 ppm, that is absent in the free compound. In the ^{13}C spectrum, protonation affects mainly to the chemical shifts of the NH neighbouring C-1 carbon, that is

Table 1

Comparison of the most significant ^1H and ^{13}C NMR data (δ, DMSO-d_6) for compounds **6** and **7** and their hydrochlorides



Compound	6	6·2HCl	7	7·HCl
NH^+	–	10.85	–	11.04
H-5	8.75	8.95	8.69	8.90
H-10	9.06	9.84	9.08	9.88
H-α	3.63	3.36	3.54	3.67
C-1	153.83	151.31	152.89	151.26
C-4	144.19	144.61	143.59	144.76
C-5	124.69	127.04	124.52	127.57
C-10	123.37	127.61	123.55	128.24
C-α	39.16	39.93	41.11	43.00

shielded in 2.52 ppm, whereas C-5 an C-10 are deshielded about 2.5–4.5 ppm. Concerning the aliphatic chains, α carbons are deshielded by 0.8 ppm in the hydrochloride, a fact also indicative of protonation at the same site. A final confirmation has been obtained by synthesizing the monobutylamino derivative **7** [Fig. 2, mp 158–160 °C, 89%, mass spectrum positive mode: 286 (M+1)] in which the piperazine ring is not present, and its corresponding monohydrochloride **7**·HCl [mp 274–276 °C, 88%, mass spectrum negative mode: 320 (M·HCl−1), %]. It was found that chemical shift variations for protons H_5 and H_{10} in the free and hydrochloride forms are closely related to those commented above for **6** and **6**·2HCl (Table 1). The same happens with carbons C-4, C-5, C-10 and C-α, that are all of them deshielded in the protonated form, whereas C-1 is shielded, in perfect agreement with results obtained for **6** and **6**·2HCl.

2.2. Functional antitumour activity

The in vitro antitumour activity for compounds **2**–**7** was determined by measurement of their cytostatic and cytotoxic properties in human tumour cell lines by XTT assays (Table 2). The human cell lines used were HT-29 (human colon adenocarcinoma), MCF-7 (human breast adenocarcinoma), NCI-H460 (human large cell carcinoma of the lung), and SF-268 (human glioblastoma). Data displayed in Table 2 show that the 1,4-bis(alkylamino) derivatives **2**–**4** present significant antiproliferative activity (10^{-6} M^{-1}). Bifunctional compound **6**·2HCl, which exhibits both hydrophobic and positively charged regions, is the most effective in inhibiting the proliferation of all the human tumour cell lines studied with an IC_{50} in the range of 10^{-7} M, with values similar to those of doxorubicin, a well established drug used in clinic. Interestingly, the mononuclear compound **7**·HCl, lacking the piperazine ring, was rather inactive. The introduction of the piperazine ring in compound **5** increased significantly the antitumour activity, and this antiproliferative activity was even further increased by the presence of both the piperazine ring and another phthalazine group in bifunctional **6**·2HCl, thus warranting more specific biological studies with this compound, that could be an interesting target for further in vitro and in vivo cytotoxic tests.

2.3. Molecular modelling

In order to gather some information about the ability of compounds **3**–**6** for intercalating into DNA, we have performed theoretical modelling studies following the methodology described in Section 3. The models obtained for the interaction of ligands **3** and **4** with DNA suggest that intercalation could be contributing to the interaction.

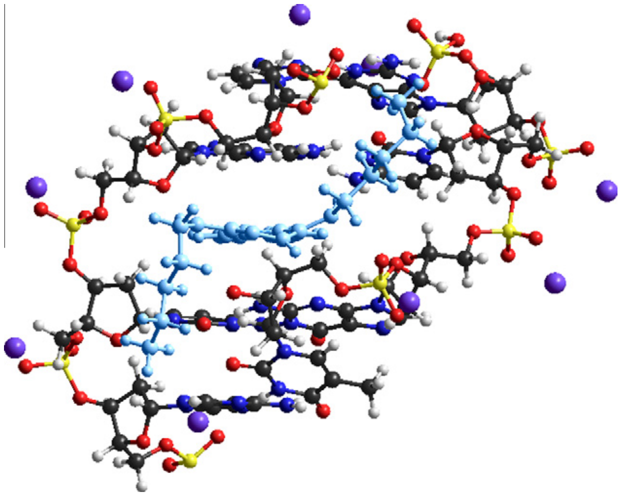


Figure 3. Image of the monoprotonated **3**–DNA complex. In this and the next drawing, DNA carbon atoms are coloured in black, nitrogens in deep blue, oxygens in red, hydrogens in pale grey, phosphorus in yellow and counterions in violet. All the atoms of the intercalating molecule are coloured in light blue.

The heteroaromatic ring of protonated **3** intercalates between cytosine-guanine base pairs while lateral chains are oriented upwards and downwards following the minor groove (Fig. 3). Theoretical stabilization for this complex is $\Delta H = -35.27$ kcal/mol, higher than that one calculated for rhodomicinone ($\Delta H = -15.67$ kcal/mol) and smaller than that one of daunomicinone ($\Delta H = -88.12$ kcal/mol). A similar behaviour is found for **4**, with $\Delta H = -29.90$ kcal/mol. In this case, although a similar intercalation mode occurs, stabilisation is lower due to the bigger volume of phenetylamine chains which difficult minor groove interaction.

With respect to the interaction of **6** with DNA, several considerations can be made. First, the geometrical features of **6** seem to be adequate for bisintercalation. Usual chain lengths for this interaction mode are known to be about 11, 14 and 18 Å for bisintercalation between two, three and four base pairs, respectively. Therefore, the calculated chain length for **6** of 12 Å, is adequate for interaction between two base pairs.[21] Since theoretical calculations of binding mode not conforming to the neighbour exclusion principle have been proposed by different authors,[22] the possibility of bisintercalation with one base pair in between was taken into consideration. Treatment of bisintercalation was done as stated previously on duplexes of five and six base pairs, obtaining much better results in the case of five base pairs. The protonation state of compound **6** at physiological pH is a crucial factor for understanding its intercalating abilities. Preliminary calculations pointed

Table 2
Inhibition of proliferation of human tumour cell lines by compounds **2**–**5**, **6**·2HCl and **7**·HCl

Compound	IC_{50} (M)			
	HT-29	MCF-7	NCI-H460	SF-268
2	$2.35 \pm 0.21 \times 10^{-6}$	$2.47 \pm 0.31 \times 10^{-6}$	$2.03 \pm 0.65 \times 10^{-6}$	$2.63 \pm 0.40 \times 10^{-6}$
3	$2.70 \pm 0.14 \times 10^{-6}$	$2.57 \pm 0.37 \times 10^{-6}$	$2.37 \pm 0.58 \times 10^{-6}$	$2.70 \pm 0.35 \times 10^{-6}$
4	$2.95 \pm 0.10 \times 10^{-6}$	$2.57 \pm 0.37 \times 10^{-6}$	$1.83 \pm 0.52 \times 10^{-6}$	$2.63 \pm 0.40 \times 10^{-6}$
5	$1.05 \pm 0.32 \times 10^{-5}$	$0.92 \pm 0.24 \times 10^{-5}$	$0.88 \pm 0.20 \times 10^{-5}$	$1.26 \pm 0.28 \times 10^{-5}$
6·2HCl	$4.97 \pm 0.10 \times 10^{-7}$	$8.63 \pm 0.80 \times 10^{-7}$	$7.42 \pm 0.60 \times 10^{-7}$	$6.65 \pm 0.20 \times 10^{-7}$
7·HCl	$>10^{-4}$	$>10^{-4}$	$8.55 \pm 0.64 \times 10^{-5}$	$8.80 \pm 0.56 \times 10^{-5}$
Doxorubucin	$2.80 \pm 0.2 \times 10^{-7}$	$3.24 \pm 0.1 \times 10^{-7}$	$2.64 \pm 0.1 \times 10^{-7}$	$2.20 \pm 0.1 \times 10^{-7}$

Data are shown as mean values ± S.D. of three independent determinations.
HT-29: human colon carcinoma cell line.
MCF-7: human breast adenocarcinoma cell line.
NCI-H460: human non-small cell lung cancer cell line.
SF.268: human glioblastoma cell line.

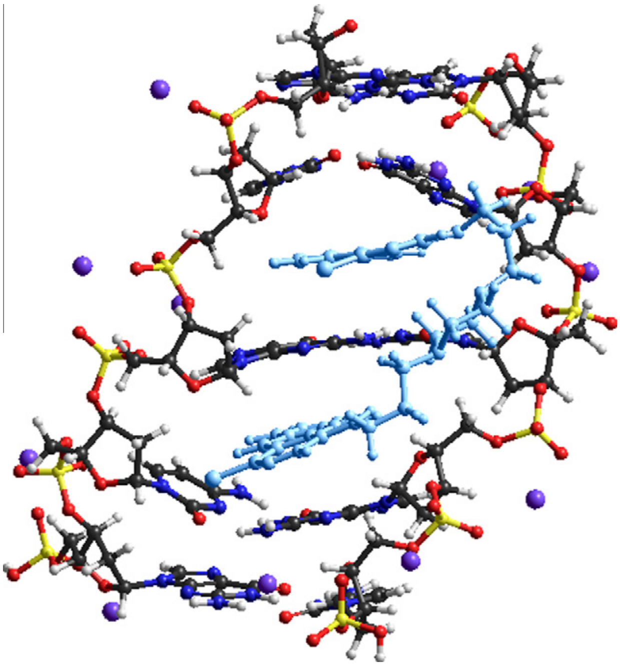


Figure 4. Image of the diprotonated **6**–DNA complex.

out that protonation of the pyridazine ring nitrogens should be favoured in all cases with respect to the piperazine ring by a difference of 5.18–7.09 kcal/mol for monoprotonatión and 12.17 kcal/mol for diprotonation. If we consider the N···H interactions in successive protonations of compound **6**, it is shown that both the first and second proton bind respectively to the two pyridazine nitrogens of the first and second benzo[*g*]phthalazine systems, creating a symmetric structure in accordance with the NMR spectra obtained for this compound. The above results were considered for calculations in the theoretical intercalation studies performed. Figure 4 displays the most stable geometries obtained for complexes of the diprotonated ligand **6** through bisintercalation on duplexes of five base pairs.

The preferred geometry found for the diprotonated **6**–DNA complex, with a ΔH value of −122.21 kcal/mol, corresponds to an arrangement of the two heterocyclic units in a 'head on' orientation that could account for the stability value found. Stabilizing hydrogen bondings between both exocyclic NH groups and their upper heterocyclic systems have been detected. Finally, the consideration of six base pairs duplexes leads to ΔH value of −42 kcal/mol, with hydrogen bondings between the base pairs and the cyclic nitrogens. All remarks pointed above are compatible with the hypothesis that compound **6**·2HCl could have DNA bisintercalating properties by interaction with base pairs in between.

2.4. DNA melting studies

After pondering the results obtained in the molecular modelling of the DNA–**6**·2HCl complex, it could be relevant to obtain some experimental information about the bisintercalating ability of bifunctional **6**·2HCl with respect to monofunctional **5**. Therefore, we have performed a DNA melting study in the presence of both ligands.

Following the method indicated in Section 3, we looked at the effect caused by the presence of **5** and **6**·2HCl on DNA melting curves measured with ct-DNA. Figure 5 plots T_m as a function of the average number of ligand molecules per ct-DNA.

The linear rise in T_m is 20.6 °C/(drug/base-pair) for **6**·2HCl, which is nearly twice the one obtained for **5**, 10.5 °C/(drug/base-pair). The increase in the slope is directly proportional to the number of occupied intercalation sites in DNA, suggesting that bisintercalation might occur for **6**·2HCl.[23] This result is in accordance with the theoretical studies commented above.

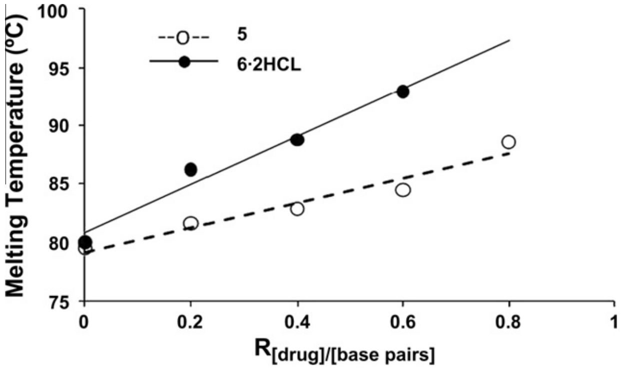


Figure 5. Melting temperatures values (°C) of (ct)-DNA upon addition of studied compounds **5** and **6**·2HCl at different ratios *r* at pH 7 (*r* = [drug]/[base-pair], [base-pair] = 7.5 × 10^{-6} M) buffer sodium cacodylate, *I* = 0.05 mol dm^{-3}).

3. Experimental

The starting materials were purchased from commercial sources (Aldrich or Fluka) and used without further purification. 1,4-Dichlorobenzo[*g*]-phtalazine was obtained from 2,3-naphthalene-dicarboxylic acid as reported previously by our group.[13] Solvents were dried using standard techniques.[24] All the reactions were monitored using thin layer chromatography (TLC) on precoated aluminium sheets of Silica Gel 60F254. Compounds were detected with UV light (245 nm). Chromatografic separations were performed on columns in the indicated solvent system using flash chromatography on silica gel (particle size 0.040–0.063 mm or standard techniques on basic aluminium oxide. Melting points were determined in a Reichert Jung hot-stage microscope. The ^{1}H and ^{13}C NMR spectra were recorded with Varian Unity XL-300, Varian Unity Inova-400, or Varian Unity 500 spectrometers at room temperature employing $CDCl_3$, CD_3OD or DMSO-d_6 as solvents. Chemical shifts are reported in ppm from tetramethylsilane (δ scale). All assignments were performed on the basis of ^{1}H–^{13}C heteronuclear multiple quantum coherence experiments (gHSQC and gHMQC). IR spectra were recorded on a Perkin Elmer 681 spectrometer. Electrospray mass spectra were recorded with a Hewlett–Packard 1100 MSD apparatus and fast atomic bombardment mass spectra with a VG Autospec spectrometer using *m*-nitrobenzyl alcohol (NBA) matrix. Elemental analyses were provided by the Departamento de Análisis, Centro de Química Orgánica 'Manuel Lora-Tamayo', CSIC, Madrid, Spain.

3.1. Synthesis of the 1,4-bis(alkylamino)benzo[*g*]-phthalazine derivatives 2–4

General procedure A: A mixture of 1,4-dichloro-benzo[*g*]phthalazine (248 mg, 1 mmol) and the corresponding amine (5 mL) was heated in an autoclave at 130 °C for 24 h. After cooling to room temperature the excess of the amine was evaporated to dryness under vacuum. The residue was treated with acetone and the solid was filtered off and dried to give the corresponding hydrochloride, which was transformed into the free compound by treatment with 5% aqueous sodium hydroxide (5 mL) and further extraction with chloroform. The organic layer was washed with water and dried. Removal of the solvent under vacuum afforded the free amine, which was purified on silica gel by flash column chromatography, using hexane/ethyl acetate/methanol (v/v 1:1:0.3) as the eluent.

3.1.1. 1,4-Bis(butylamino)benzo[*g*]phthalazine monohydrochloride (**2**·HCl)

Following the general procedure A, the reaction of 1,4-dichlorobenzo[*g*]phthalazine **1** and butylamine afforded a yellow solid identified as **2**·HCl (212 mg, 55%, mp 195–197 °C). Anal. Calcd for $C_{20}H_{26}N_4$·HCl·0.5H_2O: C, 63.74; H, 7.81; N, 14.68. Found: C, 64.87, H, 7.72; N, 14.92; IR (KBr, cm^{-1}) 3320–2660 (3240, 3100, 3000, 2940, 2920, 2850), 1625, 1575, 1545, 1425, 1360, 1325, 1155, 1025; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.10 (s, 2H, H-5, H-10), 8.33 (m, 2H, H-6, H-9), 7.97 (m, 2H, H-7, H-8), 3.67, (t, 4H, H-α), 3.49 (m, 1H, NH), 2.00 (m, 4H, H-β), 1.72, (m, 4H, H-γ), 1.22 (t, 6H, Me); ^{13}C NMR (CD_3OD, 75 MHz): δ 150.21 (C-1, C-4), 136.24 (C-5a, C-9a), 131.23 (C-7, C-8), 130.40 (C-6, C-9), 126.60 (C-5, C-10), 119.26 (C-4a, C-10a), 43.40 (C-α), 31.77 (C-β), 21.68, (C-γ), 14.51 (Me); MS-ESI (negative mode, MeOH), *m*/*z* (%): 357.1(M·HCl−1) (100).

3.1.2. 1,4-Bis(butylamino)benzo[*g*]phthalazine (2)

The hydrochloride **2**·HCl (100 mg, 0.28 mmol) was treated with 5% NaOH as stated in the general procedure and purified on silica-gel by flash column chromatography (hexane/ethyl acetate/methanol, v/v 1:1:0.3). The fraction with R_f = 0.11 afforded a hygroscopic solid, which was filtered through a small column of basic aluminium oxide using chloroform as the eluent. Removal of the solvent under vacuum yielded 74 mg (82%) of anhydrous compound **2** with mp 177–179 °C. Anal. Calcd for $C_{20}H_{26}N_4$: C, 74.53; H, 8.07; N, 17.39. Found: C, 74.78, H, 7.96; N, 17.31; IR (KBr, cm^{-1}), 3300, 3050, 2950, 2910, 2850, 1620, 1495, 1425, 1360, 1220, 1140; ^{1}H NMR (Cl_3CD, 300 MHz): δ 8.08 (s, 2H, H-5, H-10), 7.87 (m, H-6, H-9), 7.47 (m, 2H, H-7, H-8), 4.65 (br s, 2H, NH), 3.54 (t, 4H, H-α), 1.66 (m, 4H, H-β), 1.40 (m, 4H, H-γ), 0.88 (t, 6H, Me); ^{13}C NMR (Cl_3CD, 75 MHz): δ 149.74 (C-1, C-4), 133.65 (C-5a, C-9a), 128.57 (C-7, C-8), 127.70 (C-6, C-9), 121.73 (C-5, C-10), 118.45 (C-4a, C-10a), 42.09 (C-α), 31.73 (C-β), 20.49 (C-γ), 13.93 (Me); MS-EI *m*/*z* (%) 323.0 (M+1) (11), 322.0 (M) (51), 279.1 (100), 178.9 (81), 43.2 (24), 41.2 (27).

3.1.3. 1,4-Bis(pentylamino)benzo[*g*]phthalazine monohydrochloride (**3**·HCl)

Following the general procedure A, the reaction of 1,4-dichlorobenzo[*g*]phthalazine **1** and pentylamine afforded a yellow solid which was identified as **3**·HCl after crystallization from acetonitrile [309 mg, 80%, mp 185–187 °C (dec)]. Anal. Calcd for $C_{22}H_{30}N_4$·HCl·H_2O: C, 65.26; H, 7.66; N, 13.84. Found: C, 65.87, H, 7.94; N, 13.77; IR (KBr, cm^{-1}): 3429, 3269, 3054, 2956, 2929, 2859, 1625, 1551, 1466, 1354, 1148, 912, 753, 670; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.12 (s, 2H, H-5, H-10), 8.34 (m, 2H, H-6, H-9), 7.98 (m, 2H, H-7, H-8), 3.67 (t, 4H, H-α), 1.65 (m, 8H, H-β, Hγ), 1.16 (t, 3H, Me); ^{13}C NMR (CD_3OD, 75 MHz): δ 150.23 (C-1, C-4), 135.91 (C-5a, C-9a), 130.93 (C-7, C-8), 130.10 (C-6, C-9), 126.27 (C-5, C-10), 118.67 (C-4a, C-10a), 43.36 (C-α), 30.46 (C-β), 29.10 (C-γ), 23,54 (C-δ), 14.39 (Me); MS-ESI (negative mode, MeOH), *m*/*z* (%): 385.1 (100) (M·HCl-1).

3.1.4. 1,4-Bis(pentylamino)benzo[*g*]phthalazine (3)

The hydrochloride **3**·HCl (100 mg, 0.28 mmol) was treated with 0.5% NaOH as stated in the general procedure and purified on silicagel by flash column chromatography (hexane/ethyl acetate/methanol, v/v 1:1:0.3). The fraction with R_f = 0.35 (MeOH) afforded a solid, which was filtered through a small column of basic aluminium oxide using chloroform as the eluent. Removal of the solvent under vacuum yielded 68 mg (74%) of an orange compound **3** with mp 134–136 °C. Anal. Calcd for $C_{22}H_{30}N_4$·0.5H_2O: C, 73.53; H, 8.35; N, 15.59. Found: C, 73.43, H, 8,76; N, 15.41; IR (KBr, cm^{-1}): 3435, 2929, 1626, 1547, 1356, 750; ^{1}H NMR (Cl_3CD, 300 MHz): δ 8.24(s, 2H, H-5, H-10), 8.02 (m, 2H, H-6, H-9), 7.61 (m, 2H, H-7, H-8), 4.75 (br s, 2H, NH), 3.62 (t, 4H, H-α), 1.78 (m, 4H, H-β), 1.41 (m, 8H, H-γ, H-δ) 0.91 (t, 6H, Me); ^{13}C NMR (Cl_3CD, 75 MHz): δ 149.26 (C-1, C-4), 133.61 (C-5a, C-9a), 128.57 (C-7, C-8), 127.66 (C-6, C-9), 121.01 (C-5, C-10), 118.49 (C-4a, C-10a), 42.32 (C-α), 29.53 (C-β), 29.39 (C-γ), 22.57 (C-δ), 14.09 (Me); MS-ESI (positive mode, Cl_3CH) *m*/*z* (%): 351 (M+1) (100), 307 (60), 290 (45), 210 (83), 210(12), 185 (15).

3.1.5. 1,4-Bis(phenetylamino)benzo[*g*]phthalazine monohydrochloride (**4**·HCl)

Following the general procedure A, the reaction of 1,4-dichlorobenzo[*g*]phthalazine **1** and phenetylamine afforded a yellow solid which after being crystallized from acetonitrile was identified as **4**·HCl (295 mg, 65%) [mp 143–145 °C (dec)]. Anal. Calcd for $C_{28}H_{26}N_4$·HCl·0.5H_2O: C, 72.49; H, 6.04; N, 12.08. Found: C, 72.06; H, 6.53; N, 12.24; IR (KBr, cm^{-1}): 3054, 3025, 2925, 2862, 1625, 1549, 1453, 1351, 1197, 1114, 1031, 893, 750, 699; ^{1}H NMR (CD_3OD, 300 MHz): δ 9.12 (br s, 2H, H-5, H-10), 8.31 (br s, 2H, H-6, H-9), 7.90 (br s, 2H, H-7, H-8), 7.47 (m, 8H, H-2′, H-3′), 7.38 (2H, H-4′), 3.93 (br s, 4H, H-α), 3.50 (m, 2H, NH), 3.30 (t, 4H, H-β); ^{13}C NMR (CD_3OD, 75 MHz): δ 150.52 (C-1, C-4), 141.5 (C-1′), 136.00 (C-5a,C-9a), 131.03 (C-7, C-8), 130.12 (C-6, C-9), 129.94 (C-2′), 129.59 (C-3′), 127.80 (C-4′), 127.57 (C-5, C-10), 118.86 (C-4a, C-10a), 44.77 (C-α), 35.52 (C-β); MS-ESI (negative mode, MeOH), *m*/*z* (%): 453.1 (M·HCl-1) (100).

3.1.6. 1,4-Bis(phenetylamino)benzo[*g*]phthalazine (4)

The hydrochloride **4**·HCl (100 mg, 0.22 mmol) was treated with 0.5% NaOH as stated in the general procedure and purified on silicagel column by flash chromatography (hexane/ethyl acetate/methanol, v/v 1:1:0.3). The fraction with R_f = 0.46 (MeOH) afforded a solid, which was filtered through a small column of basic aluminium oxide using chloroform as the eluent. Removal of the solvent under vacuum yielded 72 mg (78%) of an orange compound **4** with mp 65–67 °C. Anal. Calcd for $C_{28}H_{26}N_4$·0.25H_2O: C, 79.52; H, 6.27; N, 13.25. Found: C, 79.60, H, 6.22; N, 13.47; IR (KBr, cm^{-1}): 3435, 1622, 1496, 1347, 748, 699. ^{1}H NMR (Cl_3CD, 300 MHz): δ 8.15 (s, 2H, H-5. H-10), 7.96 (m, 2H, H-6, H-9), 7.80 (m, 8H, H-2′, H-3′), 7.59 (m, 2H, H-7, H-8), 7.22 (m, 2H, H-4′), 4.78 (br s, 2H, NH), 3.96 (c, 4H, H-α), 3.12 (t, 4H, H-β); ^{13}C NMR (Cl_3CD, 75 MHz): δ 149.13 (C-1, C-4), 140.00 (C-1′), 133,62 (C-5a, C-9a), 129.00 (C-2′), 128.59 (C-7, C-8), 128.53 (C-3′), 127.74 (C-6, C-9), 126.25 (C-4′), 120.97 (C-5, C-10), 118.44 (C-4a, C-10a), 43.00 (C-α), 35.44 (C-β); MS-ESI (positive mode, Cl_3CH): *m*/*z* (%) 419 (M+1) (100).

3.2. Synthesis of the 1-alkylamino-4-chlorobenzo[*g*]-phthalazine derivatives

General procedure B: To a refluxed suspension of 1,4-dichlorobenzo[*g*]phthalazine (248 mg, 1 mmol) and anhydrous potassium carbonate (1.38 g, 10 mmol) in anhydrous acetonitrile (130 mL) a solution of the corresponding amine in anhydrous acetonitrile (50 mL) was slowly added. The reaction was further refluxed and monitored by TLC plates until completion (7 days).

3.2.1. Synthesis of compounds 5 and 6

Following method B, 1,4-dichlorobenzo[*g*]phthalazine was made react with 1,4-bis(propylamino)piperazine (200 mg, 1 mmol). After cooling to room temperature, a yellow solid containing the reaction products and potassium carbonate was separated by filtration and extracted with chloroform. The organic solution was evaporated under reduced pressure to give a brown residue which was purified on silica gel by flash column chromatography (chloroform/ethanol/25% ammonium hydroxide v/v 3:1:0.1). Following monitorization by TLC, the appropriate fractions were combined to give two products with R_f 0.12 and 0.79.

3.2.1.1. 1-[3-(4-Aminopropylpiperazin-1-yl)propyl-amino]-4-chlorobenzo[*g*]phthalazine (5). The most retained fraction (R_f = 0.12) afforded 190 mg (46%) of an orange solid with mp 250–252 °C. Anal. Calcd for $C_{22}H_{29}N_6Cl{\cdot}H_2O$: C, 61.32; H, 7.2; N, 19.51. Found: C, 61.82; H, 7.33; N, 19.55; IR (KBr, cm^{-1}), 3435, 2932, 1630, 1555, 1505, 1431, 1156; ^1H NMR ($CDCl_3$, 500 MHz), δ 8.59 (s, 1H, H-10), 8.45 (s, 1H, H-5), 8.08 (m, 2H. H-6, H-9), 7.63 (m, 2H, H-7, H-8), 3.78 (t, 2H; H-α), 2.83 (m, 2H, H-α′), 2.63 (m, 10H, 2H-γ, 4H-δ, 4H-δ′), 2.55 (t, 2H, H-γ′), 1.94 (m, 2H, H-β), 1.72 (m, 2H, H-β′); ^{13}C NMR ($CDCl_3$, 125 MHz), δ 159.83 (C-1), 145.50 (C-4), 134.24, 134.35 (C-5a, C-9a), 129.17 (C-6, C-9), 128.25 (C-7, C-8), 125.59 (C-5), 122.90 (C-4a), 121.71 (C-10), 118.40 (C-10a), 58.63 (C-γ), 56.82 (C-γ′), 53.63, 53.58 (C-δ, C-δ′), 43.35 (C-α), 40.83 (C-α′), 29.13 (C-β′), 23.77 (C-β); MS-FAB, *m*/*z* (%): 413 (M+1, 100), 270 (80), 154 (20).

3.2.1.2. 1,4-Bis-{3-[*N*-(4-chlorobenzo[*g*]phtalazin-1-yl)-amino-propyl]}piperazine (6). The less retained fraction (R_f = 0.79) gave 122 mg (20%) of a yellow solid with mp 244–246 °C. Anal. Calcd for $C_{34}H_{34}N_8Cl_2{\cdot}1.5H_2O$: C, 62.57; H, 5.67; N, 17.17. Found: C, 62.52; H, 5.95; N, 16.94; IR (KBr, cm^{-1}), 3435, 3277, 2888, 2800, 1622, 1544, 1518, 1417, 1098, 887; ^1H NMR (DMSO-d_6, 500 MHz), δ 9.06 (s, 2H, H-10), 8.75 (s, 2H, H-5), 8.36 (m, 2H, H-6), 8.16 (m, 2H, H-9), 7.78 (m, 4H, H-7, H-8), 3.63 (m, 4H, H-α), 3.1–2.65 (very broad signal, 8H, H-δ), 2.62 (m, 4H-γ), 1.98 (m, 4H, H-β); ^{13}C NMR (DMSO-d_6, 125 MHz), δ 153.83 (C-1), 144.19 (C-4), 134.07, 134.01 (C-5a, C-9a), 129.10 (C-6), 128.75 (C-9), 128.59 (C-7, C-8), 124.69 (C-5), 123.37 (C-10), 122.16 (C-10a), 117.81 (C-4a), 55.45 (C-γ), 51.96 (C-δ), 39.16 (C-α), 26.04 (C-β); MS-ESI (positive mode, MeOH) *m*/*z* (%): 625 (M+1, 18), 583 (22), 425 (18), 423 (46), 384 (34), 271 (23), 270 (100).

Following the general procedure B, 1,4-dichlorobenzo[*g*]phthalazine (30 mg, 0.12 mmol) was made react with compound **5** (50 mg, 0.12 mmol). After cooling to room temperature, potassium carbonate was eliminated by filtration, the organic solution evaporated under reduced pressure, and the residue purified on silicagel by flash column chromatography (chloroform/ethanol/aq 25% ammonium hydroxide v/v 3:1:0.1). The combined fractions with R_f = 0.79 gave an additional amount of a yellow solid identified as compound **6** (12 mg, 16%).

3.2.1.3. 1,4-Bis-{3-[*N*-(4-chlorobenzo[*g*]phtalazin-1-yl)-amino-propyl]}piperazine hydrochloride (6·2HCl). A solution of 1,4-bis{3-[N-(4-chlorobenzo[*g*]-phthalazin-1-yl)aminopropyl]} piperazine, **6**, (42 mg, 0.067 mmol) in 10 mL of methanol and 5 mL of chloroform was prepared, and a solution of 0.4 mL of freshly distiled acetyl chloride in 15 mL of methanol, maintained under argon atmosphere and cooled down to 0 °C for 30 min, was added dropwise. At the end of the addition the solution looked turbid and, after stirring for 1 h at 0 °C, the presence of a heavy precipitate was evident. After that, 15 mL of ether were added. One hour more of stirring and the reaction mixture was filtered under argon to give 41.5 mg (88%) of a pale brown solid that was identified as the dihydrochloride of compound **6**. Mp 250–252 °C. Anal. Calcd for $C_{34}H_{36}\ Cl_4N_8{\cdot}0.8Cl_3CH{\cdot}8H_2O$: C, 44.02; H, 5.68; N, 12.06; Cl, 24.43. Found: C, 43.70; H, 5.60; N, 12.48, Cl, 24.54. IR (KBr, cm^{-1}), 3435, 2923, 2889, 2730, 2622, 2510, 1622, 1462, 1379, 1191, 1163, 1124; 974, 758, 664. ^1H NMR (DMSO-d_6, 500 MHz): δ 10.85 (broad signal (2NH$^+$), 9.84 (s, 2H, H-10), 8.95 (s, 2H, H-5), 8.48 (s, 2H, H-6), 8.24 (m, 2H, H-9), 7.94 (m, 4H, H-7, H-8), 3.83 (br s, 4H, H-α), 3.62 (m, 8H, H-δ), 3.36 (m, 4H, H-γ) 2,26 (br s, 4H, H-β); ^{13}C NMR (DMSO-d_6, 125 MHz), δ 151.31 (C-1), 144.61 (C-4), 135.04 (C-9a), 133.08 (C-5a), 130.33 (C-7,8), 129.41 (C-6), 128.89 (C-9), 127.61 (C-10), 127.04 (C-5), 121.55 (C-9a), 117.82 (C-4a), 52.50 (C-γ), 47.83 (C-δ), 39.93 (C-α), 21.98 (C-β); ESI (negative mode, MeOH) *m*/*z* (%): 698.3 (M·2HCl-1, 35), 659.2 (M·HCl-1)(55), 505.2 (30), 475.2 (100). ESI (positive mode, MeOH) *m*/*z* (%): 625.5 [(M+1)−2HCl] (60), 270.1 (100).

Alternatively, the double hydrochloride of **6** was also obtained as follows: a solution of 1,4-bis-(propylamino)piperazine (200 mg, 0.75 mmol) in anhydrous acetonitrile (50 mL) was slowly added to a suspension of 1,4-dichlorobenzo[*g*]phthalazine (1 mmol) in anhydrous acetonitrile (130 mL), and one drop of HCl 6 N was added. The mixture was further refluxed and monitored by TLC plates until the reaction was completed. The organic solution was evaporated under reduced pressure and the residue was purified on silica gel by flash column chromatography (chloroform/ethanol/25% ammonium hydroxide v/v 3:1:0.1). After monitorization by TLC, the appropriate fractions were combined to give a brown solid of R_f = 0.84 which, after drying under vacuum yielded 104 mg (29%) of **6**·2HCl, with mp 250 °C (dec). MS-ESI (positive mode, MeOH) *m*/*z* (%): 625.2363 [(M·2HCl +1)−2HCl] (38), 356.1647 (77), 270.0864 (100), 230.0481 (38).

3.2.2. 1-Butylamino-4-chlorobenzo[g]phthalazine 7

Following the general procedure B, 1,4-dichlorobenzo[*g*]phthalazine (249 mg, 1 mmol), and butylamine (366 mg, 5 mmol)(0.49 mL) were reacted. After cooling to room temperature, potassium carbonate was eliminated by filtration, and the organic solution evaporated under reduced pressure. The brown residue was purified on silicagel column by flash chromatography (Cl_3CH/EtOH, v/v 30:1). The appropriate fractions (R_f 0.37) monitored by TLC were combined to give **7** (253 mg, 89%) as a yellow solid with mp 158–160 °C. Anal. Calcd for $C_{16}H_{16}N_3Cl$: C, 67.48; H, 5.27; N, 14.76. Found: C, 67.55; H, 5.55; N,14.64; IR (KBr, cm^{-1}), 3422, 3271, 2956, 2869, 1564, 1520, 1423, 1379, 1288, 1159, 1119, 1069, 893, 745, 668; ^1H NMR (DMSO-d_6): δ 9.08 (s, 1H, H-10), 8.69 (s, 1H, H-5), 8.32 (br s, H-9), 8.11 (br s, 1H, H-6), 7.66 (m, 2H, H-7, H-8), 5.5 (br signal, 1H, NH), 3.54 (br s, 2H, H-α), 2.49 (br s, 2H, H-β), 1.36 (br s, 2H, H-γ), 0.90 (t, 3H, Me); ^{13}C NMR (Cl_3CD): δ 152.89 (C-1), 143.59 (C-4), 133.35, 133.62 (C-5a. C-9a), 128.72 (C-9), 128.29 (C-6), 128.55, 128.47 (C-7,C-8), 124.52 (C-5) 123.55 (C-10), 121.67 (C-10a), 117.40 (C-4a), 41.11 (C-α), 27.47 (C-β), 21.58 (C-γ), 13.53 (CH_3); MS-ESI (positive mode MeOH) *m*/*z* (%): 286 (M+1) (100).

3.2.3. 1-Butyl-4-chlorobenzo[g]phthalazine hydrochloride 7·HCl

A solution of 1-butyl-4-chlorobenzo[*g*]phthalazine **7** (86 mg, 0.3 mmol) in 10 mL of methanol and 5 mL of chloroform was prepared, and a solution of 0.5 mL of freshly distiled acetyl chloride in 20 mL of methanol, maintained under argon atmosphere and cooled down to 0 °C for 30 min, was added dropwise. After finishing addition the solution was stirred for 1 h at 0 °C, and 80 mL of ether were added. After stirring for 1.5 h more the organic solution was partially concentrated until a precipitate was formed. The reaction mixture was filtered under argon to give the titled compound **7**·HCl (33 mg, 88%) as a pale brown solid with mp 274–276 °C. Anal. Calcd for $C_{16}H_{17}N_3Cl_2{\cdot}H_2O$: C, 56.48; H, 5.63; N, 12.35; Cl, 20.84. Found: C, 66.66; H, 5.68; N, 12.69; Cl, 20.84. IR (KBr, cm^{-1}): 2435, 3159, 3016.48, 2958, 2928, 2871, 2747, 1620, 1463, 1378, 1289, 1161, 1122, 971, 893, 753, 661; ^1H NMR (DMSO-d_6, 400 MHz): δ 11.04 (low s, 2H, $^+$NH), 9.88 (s, 2H, H-10), 8.90 (s, 2H, H-5), 8.46 (m, 2H, H-6), (8.23 (m, 2H, H-9), 7.93 (m, 4H, H-7, H-8), 3.67 (m, 4H, H-α), 1.76 (m, 4H, H-β), 1.49 (m, 4H, H-γ), 0.95 (t, 6H, Me); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 151.26 (C-1), 144.74 (C-4), 135.42 (C-9a), 134.41 (C-5a), 131.10 (C-7), 130.82 (C-8), 129.87 (C-6), 129.42 (C-9), 128.24 (C-10), 127.57 (C-5), 122.03 (C-10a), 118.28 (C-4a), 43.00 (C-α), 29.83 (C-β), 19.94 (C-γ), 14.03 (Me). MS-ESI (negative mode MeOH) *m*/*z* (%): 320 (M−1) (100); MS-ESI (positive mode) *m*/*z* (%): 286 [(M+1)−HCl] (100).

3.3. Cell culture

HT-29 (human colon adenocarcinoma), MCF-7 (human breast adenocarcinoma), NCI-H460 (human non-small cell lung carcinoma), and SF-268 (human glioblastoma) cell lines were cultured in DMEM culture medium containing 10% (v/v) heat-inactivated faetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were periodically tested for *Mycoplasma* infection and found to be negative.

3.4. Cell growth inhibition assay

The effect of the distinct compounds in the proliferation of human tumour cell lines was determined as previously described[25] by using the XTT (sodium 3,3'-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulphonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Cells (1.5×10^3 in 100 μL) were incubated in DMEM culture medium containing 10% heat-inactivated FBS, in the absence and in the presence of the indicated compounds at a concentration range of 10^{-4} to 10^{-9} M, in 96-well flat-bottomed microtiter plates, and following 72 h of incubation at 37 °C in a humidified atmosphere of air/CO_2 (19/1) the XTT assay was performed. Measurements were done in triplicate, and the IC_{50} (50% inhibitory concentration) value, defined as the drug concentration required to cause 50% inhibition in the cellular proliferation with respect to the untreated controls, was determined for each compound.

3.5. Molecular modelling

Models were built by using the Hyperchem 7.5 (Hypercube Inc.)[26] package capabilities. Monointercalation was studied on tetramer duplexes following the Newlin et al. methodology and looking for specificity among the different base pair sequences.[18,27] Treatment of bisintercalation was done in a similar way on duplexes of five and six base pairs, as the chain length of C and D allows intercalation with one or two base pairs in between. We selected the AMBER method[28] modified by the inclusion of the appropriate parameters.[29] When available, the parameters came from analogous parameters used in the literature. All others were developed following Kölman and Hopfinger procedures.[29,30] The equilibrium bond length and angle values came from experimental ones on reasonable reference compounds. Solvent effects were simulated by the use of a distance-dependent dielectric function of $\varepsilon = 4r$ in all calculations. In order to achieve electrical neutrality an appropriate number of Na^+ counterions were included, placed at 6 Å distance from each phosphate-oxygen bisector, being initially energy-minimized using 500 and 2000 cycles of steepest descent and conjugate gradients.

Model intercalation sites were generated by separation of the bases until 680 pm, and molecular mechanics energy minimization was then used to relieve any steric distortion in the chains, following the same procedure. Counterions positions were modified accordingly. Distance restraints with a force constant of 25 kcal mol^{-1} A^{-2} were applied to nucleotides affected during this process, in order to consider the Watson-Crick hydrogen bonds, and convergence was achieved at 0.1 kcal mol^{-1} A^{-1}.

Models for each synthesized compound were constructed using standard geometry and standard bond lengths. Atom potentials were assigned according to parameters defined within the AMBER force-field and lowest energy conformers were located by performing simulated annealing. These conformers were used for docking. Then, each drug structure was manually docked into the tetramer DNA duplex intercalation site, looking for optimal orientations by computing non-bonded interactions between the reactants. Alterations to ligand side chain conformations were performed in order to minimize close intermolecular contacts with the host.

The complexes were refined by progressively minimizing their potential energy: only the intercalator drug atoms were allowed to move in the first place, and finally the whole systems were relaxed, using the Polak-Ribiere algorithm (conjugate gradient) until an energy convergence criterion of 0.1 kcal mol^{-1} A^{-1} was reached.

To verify that the placement of the intercalator resulted in a structure with a low energy minimum on the potential surface for each complex, the intercalator was translated from its minimized position by ±100 pm along the principal axis of the chromophore and the resulting structures were optimized using our minimization protocol.

The intercalation energies were calculated as the sum of the energy required for opening of the DNA sequence (ΔE_i) and the intermolecular interaction energy between the intercalator and DNA (ΔE_c)

$$E_{\text{binding}} = E_{\text{complex}} - (E_{\text{oligomere}} + E_{\text{compound}}) = \Delta E_c$$

Solvation energy contributions were not included in binding calculations.

3.6. DNA melting measures

Calf thymus DNA (ct-DNA) was purchased to Aldrich. The polynucleotide was dissolved in sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}, pH 7.0. The calf thymus ct-DNA was additionally sonicated and filtered through a 0.45 μm filter.[31] Polynucleotide concentration was determined spectroscopically[32] as the concentration of base pairs (7.5×10^{-6} M). Electronic absorption spectra were recorded with an Agilent 8453 UV–vis spectrophotometer using (1 cm) quartz cuvettes.

Thermal melting curves for ct-DNA were determined by following the absorption change at 260 nm as a function of temperature of solutions in which increasing amounts of **5** and **6**·2HCl were added to ct-DNA.[32] The absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalised. T_m values are the midpoints of the transition curves, determined either from the maximum of the first derivative or graphically, by a tangent method.[33] ΔT_m values were calculated by subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least three measurements; the error in ΔT_m was estimated to be ±0.5 °C. In the two studied system (5-ct-DNA and 6-ct-DNA) molar ratios r = [drug]/[base−pair] greater than 1.0 led to precipitation.

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