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Synthesis, biological evaluation and DNA binding properties of novel mono and bisnaphthalimides

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A novel series of mono and bisnaphthalimides was synthesized and their antiproliferative activities were evaluated against three tumor cell lines. Bisnaphthalimides **3** and **4**, bearing a pyrazine ring fused to the naphthalimide system, showed activities in the order of 10^{-8} μM, similar to elinafide. DNA binding properties and the ability to induce DNA damage were studied for some of the most active compounds.

Introduction

Over the past two decades we have designed and synthesized a large range of DNA-targeted antitumor naphthalimides.[1] Two of them, amonafide and elinafide (Fig. 1), have been selected for phase I and II clinical trials.[2,3] Elinafide is highly effective against tumor xenographts *in vivo*[3] and is reported to bisintercalate DNA *via* the major groove.[4,5]

Substitution of the naphthalene moiety by an anthracene system has led to azonafide[6] and bibenoline[7] (Fig. 1), compounds that have shown an interesting *in vitro* and *in vivo* antitumor profile.

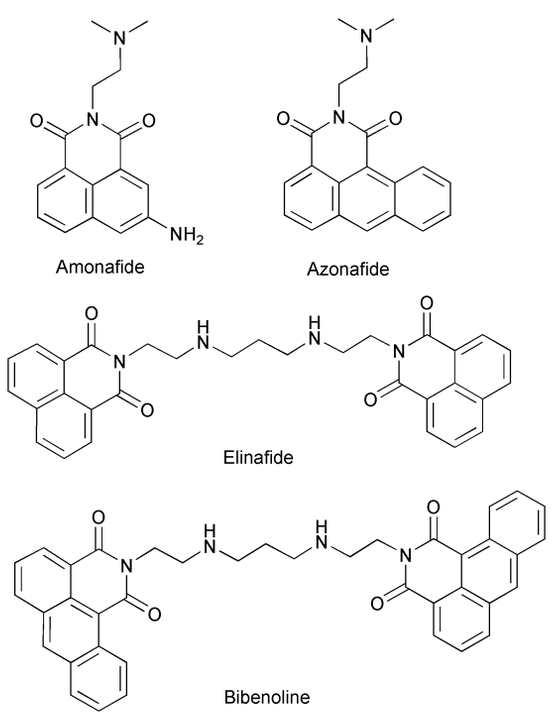

Fig. 1

In a recent study,[8] we synthesized a new series of mono and bisnaphthalimides, where an imidazole ring was fused to the naphthalene moiety, in an attempt to broaden the scope of this kind of highly active compound. However, no significant improvement in the antitumor activity was observed and dimerization of the chromophore led to a considerable decrease in activity. This prompted us to change the imidazole ring to a π-deficient pyrazine ring. Here, we report the synthesis and biological evaluation of a new series of mono and bisintercalating agents related to amonafide and elinafide, where a pyrazine heterocycle has been fused to the naphthalimide system (**1**–**6**) (Fig. 2). Compounds **2**, **5** and **6**, bearing two electron withdrawing trifluoromethyl groups, were selected in order to obtain highly electron deficient chromophores, where the stacking interactions with the DNA bases could be enhanced, favoring the intercalation process. Finally, mononaphthalimide **7** is an analogue of amonafide where an additional amino group has been introduced. This compound and the corresponding bisnaphthalimides **8** and **9**, were selected because they are readily accessible from one of the intermediates in the synthesis of pyrazinonaphthalimides.

Results and discussion

Chemistry

Mononaphthalimides **1**, **2** and **7** were synthesized by treatment of the corresponding anhydride with an excess of *N,N*-dimethylethane-1,2-diamine in ethanol at reflux temperature. Bisnaphthalimides **3**–**6** were obtained by a similar procedure, starting from the corresponding polyamine and anhydride in a 1 : 2 ratio. In the synthesis of **8** and **9** an excess of amine was necessary for the reaction to be completed. However, formation of the corresponding mononaphthalimides was not observed in the crude reaction and the bisnaphthalimides could be isolated in good yields. *N,N′*-bis(2-aminoethyl)propane-1,3-diamine used in the synthesis of bisnaphthalimides **3**, **5** and **8** was commercially available, and *N,N′*-bis(2-aminoethyl)-*N,N′*-dimethylpropane-1,3-diamine, used in the synthesis of

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Table 1 Antiproliferative activities of pyrazine and diaminonaphthalimides

Compds	HT-29[a] IC_{50}/μM	HeLa[b] IC_{50}/μM	PC-3[c] IC_{50}/μM
1	1.05	1.95	4.60
2	2.77	3.86	4.14
3	0.02	0.20	0.53
4	0.02	0.08	0.80
5	1.27	1.34	7.80
6	4.16	6.58	3.19
7	1.62	1.89	1.46
8	0.42	0.45	4.06
9	0.06	0.09	0.18
Amonafide	4.67	2.73	6.38
Elinafide	0.02	0.07	0.32

[a] Human colon carcinoma cell line. [b] Human cervical carcinoma cell line. [c] Human prostate carcinoma cell line.

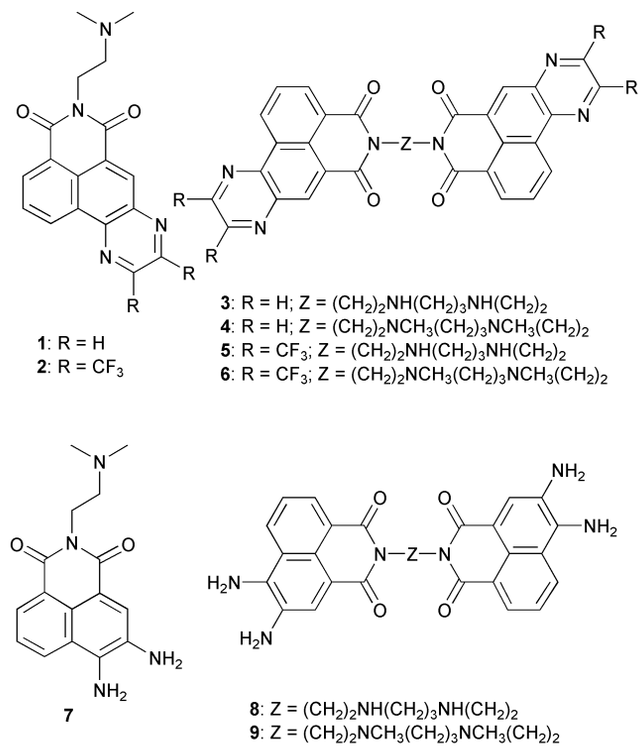
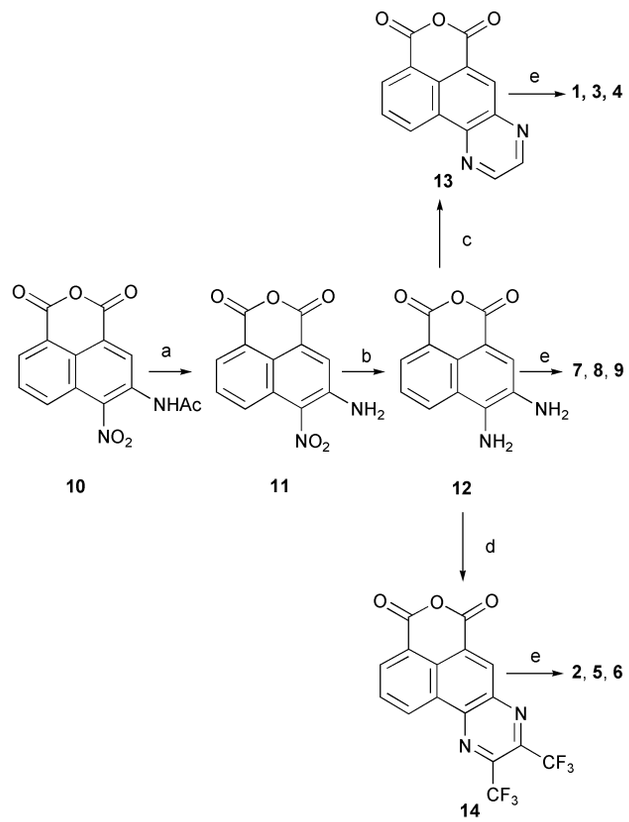


Fig. 2

bisnaphthalimides **4**, **6** and **9**, was obtained by the method previously described.[8] All these compounds were isolated and purified by flash chromatography on silica gel or by recrystallization and were obtained with yields that vary from high (98%) to moderate (55%). Starting anhydrides **13** and **14** were synthesized from the previously described 3-acetylamino-4-nitronaphthalene-1,8-dicarboxylic anhydride (**10**),[8] as depicted in Scheme 1. Thus, hydrolysis of **10** with concentrated H_2SO_4 in ethanol, followed by hydrogenation with Pd/C (10%) in DMF gave 3,4-diaminonaphthalene-1,8-dicarboxylic anhydride (**12**). This compound was converted into anhydrides **13** and **14** by reaction with glyoxal and 1,1,1,4,4,4-hexafluorobutane-2,3-dione respectively.

Biological evaluation

Mono and bisnaphthalimides **1**–**9** were evaluated for *in vitro* cytotoxicity against human colon carcinoma (HT-29), human cervical carcinoma (HeLa) and human prostate carcinoma (PC-3) cell lines. Antiproliferative activities were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results are summarized in Table 1 and compared with the activity of amonafide and elinafide. Pyrazinonaphthalimides (compounds **1**–**6**) were tested as their methanosulfonate salts, while diaminonaphthalimides (compounds **7**–**9**) were tested as free bases.



Scheme 1 (a) concentrated H_2SO_4, EtOH, Δ, overnight (95%); (b) H_2, Pd/C (10%), DMF, overnight (97%); (c) glyoxal (40%, water), Δ, 24 h (95%); (d) 1,1,1,4,4,4-hexafluorobutane-2,3-dione, DMF, 0 °C, 24 h under argon (20%); (e) for **1**, **2** and **7**: $NH_2(CH_2)_2N(CH_3)_2$; for **3**, **5** and **8**: $NH_2(CH_2)_2NH(CH_2)_3NH(CH_2)_2NH_2$; for **4**, **6** and **9**: $NH_2(CH_2)_2$-$NCH_3(CH_2)_3NCH_3(CH_2)_2NH_2$.

Monomeric naphthalimides show a pattern of activity similar to amonafide. Dimerization of compound **2** has not led to an increase in the antiproliferative activity. In this case, bisintercalation could be impeded by the steric hindrance produced by the presence of two trifluoromethyl groups in the chromophore. In contrast, the antitumor activity of pyrazinonaphthalimide **1** increases substantially with dimerization, showing values of IC_{50} very similar to the values found for elinafide in the same experimental conditions. Some of these compounds were selected to carry out further studies, as shown below.

Finally, diamino derivative **9** also shows improved activities in the order of 10^{-8} M. In this case methylation of the amino groups in the linking chain has led to an increase of activity, as can be seen by comparison with the unmethylated compound **8**.

DNA binding properties

The DNA binding properties of compound **1** as a model of a monointercalator and **3** as a model of a bisintercalator were studied by UV–VIS spectrometry and viscosimetry. Addition of these compounds to a sample of sonicated calf thymus DNA in tris–HCl (pH = 6.9) induces hypochromic and bathochromic shifts, the shift being higher as the temperature decreases. This is the expected behavior for an intercalator.[9] Melting temperature (Tm) measurements, carried out at a drug : DNA ratio of 0.1, gave monophasic melting curves with ΔTm values ($\Delta T\text{m} = T\text{m}^{\text{complex}} - T\text{m}^{\text{DNA}}$) of 3.9 and 19.6 °C for **1** and **3**, respectively (and 15.6 °C for **4**). The dimeric compound stabilizes duplex DNA against heat denaturation considerably more strongly than the monomer. This is a strong indication that the two moieities of **3** (and **4**) are engaged in the DNA binding process.

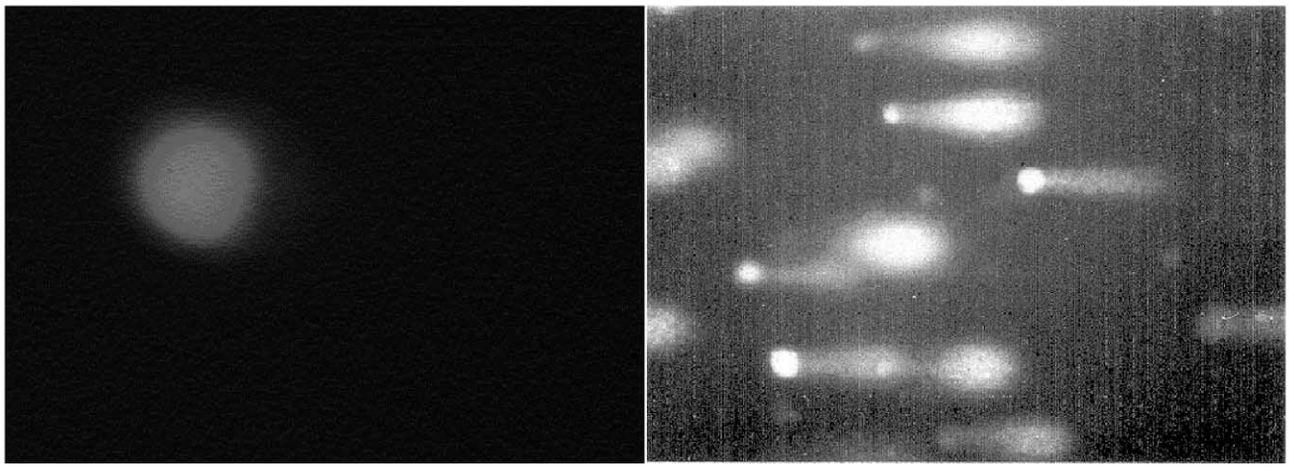


Fig. 3 Single-cell gel electrophoresis assay for a negative control (left) and for compound **3** (right).

This conclusion was also supported by viscosimetric measurements. The relative length increase L : L_0 of the drug–DNA complexes for compounds **1** and **3** were measured as a function of the molar ratio of added compound to the sonicated calf thymus DNA (*r*), giving slopes of 1.3 and 2.7, which is the expected behavior for a mono and bifunctional intercalator respectively.[10,11]

Comet assay

To further evaluate the mechanism of action, compound **3** was selected for the assay of single-cell gel electrophoresis in HT-29 cells (comet assay). The comet assay detects DNA damage in individual cells embedded in agarose. The test[12] is based on the property of negatively charged DNA fragments to migrate when an electric field is applied to the gel after cell lysis. Doxorubicin was chosen as a positive reference and PBS (phosphate buffered saline, pH = 7.4) was used as a negative control. One hour after the treatment, the samples were observed using a fluorescence microscope and the DNA damage of compound **3** and no DNA damage in the negative control are shown in Fig. 3. This result suggests that the antitumor activity of these bisnaphthalimides may be related to their ability to induce DNA damage and this prompted us to examine their effects on *topoisomerases*.

DNA *topoisomerase* inhibition

The two drugs **1** and **3** inhibit DNA relaxation by *topoisomerase I* but they do not stabilize DNA–enzyme covalent complexes. The band corresponding to the nicked DNA increases considerably in the presence of the reference drug camptothecin whereas no such effect is observed with **1** or **3** (Fig. 4). A much larger retardation of the electrophoretic mobility of DNA band is observed with the bisnaphthalimide **3** compared to the monomeric analogue **1**. This reflects the higher DNA binding affinity of the dimer compared to the monomer, as expected from the *T*m measurements. With the type *II* enzyme, a band of linear DNA is detected with the reference inhibitor etoposide and **1** which therefore can be considered a *topoisomerase II* poison, as is amonafide.[13] In contrast, the bisnaphthalimide **3** does not stabilize DNA–*topoisomerase II* covalent complexes. As with *topoisomerase I*, it induces a marked shift of the plasmid, again reflecting tight binding to DNA, but there is no stabilization of *topoisomerase*–DNA cleavable complexes with **3**.

Conclusions

In summary, pyrazinonaphthalimides have shown to be interesting chromophores in the design of new mono and bisintercalators with a high antitumor activity. Further *in vivo* studies of some of these compounds will be carried out.

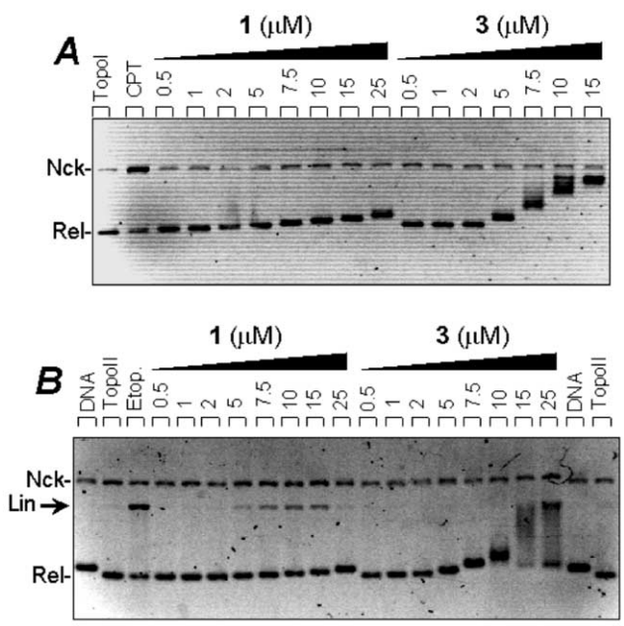


Fig. 4 Inhibition of human *topoisomerases I* (A) and *II* (B) in the presence of the mono (**1**) and bisnaphthalimide (**3**) compounds at the indicated concentration (M). Camptothecin (CPT) and Etoposide (Etop.) were used as positive controls (50 μM each) for the poisoning of *topoisomerases I* and *II*, respectively. Nck, nicked; Lin, linear; Rel, relaxed DNA.

Experimental section

General methods

Melting points (uncorrected) were determined on a Stuart Scientific SMP3 apparatus. Infrared (IR) spectra were recorded with a Perkin-Elmer 1330 infrared spectrophotometer. ^{1}H and ^{13}C NMR: δ were recorded on a Bruker 300-AC instrument. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in Hertz. Mass spectra were run on a HP 5989A spectrometer. Elemental analyses (C, H, N) were performed on a Perkin-Elmer 2400 CHN apparatus at the Microanalyses Service of the University Complutense of Madrid. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates. Unless stated otherwise, starting materials used were high-grade commercial products.

3-Amino-4-nitronaphthalene-1,8-dicarboxylic anhydride (11)

A mixture of **10**[8] (500 mg, 1.67 mmol) and concentrated H_2SO_4 (2.3 cm^3) in EtOH (15 cm^3) was heated at reflux for 24 h. The resulting mixture was poured into water and ice. The precipitated solid was filtered, washed with water and dried. Recrystallization from AcOEt gave **11** (409 mg, 95%) as a brown solid, mp 238–240 °C; ν_{max}(KBr)/cm^{-1} 3460, 3350, 1770 and 1730; δ_H (300 MHz; DMSO-d_6; Me_4Si) 8.64 (1 H, d, *J* 8.5, ArH), 8.23 (1 H, s, ArH), 8.21 (1 H, d, *J* 9.2, ArH), 7.98 (2 H, br s, NH_2) and 7.88 (1 H, m, ArH); δ_C (75 MHz; DMSO-d_6; Me_4Si) 160.3 (CO), 159.5 (CO), 144.6, 130.2, 128.2, 127.5, 127.0, 126.9, 126.8, 124.3, 122.4 and 119.5 (ArH).

3,4-Diaminonaphthalene-1,8-dicarboxylic anhydride (12)

A mixture of **11** (900 mg, 3.49 mmol) and 10% Pd/C (45 mg) in DMF (25 cm^3) was shaken in a Parr hydrogenator under hydrogen at 50 Psi pressure for 24 hours. The catalyst was then filtered off and washed with DMF. The filtrate was concentrated, and water was added. The precipitate was then filtered and washed with water. Recrystallization from DMF gave **12** (774 mg, 97%) as a red solid, mp >300 °C (lit.,[14] mp >300 °C); ν_{max}(KBr)/cm^{-1} 3420, 3360, 1730 and 1680; δ_H (300 MHz; DMSO-d_6; Me_4Si) 8.57 (1 H, d, *J* 8.6, ArH), 8.19 (1 H, d, *J* 6.7, ArH), 7.87 (1 H, s, ArH), 7.58 (1 H, m, ArH), 6.88 (2 H, br s, NH_2) and 5.27 (2 H, br s, NH_2); δ_C (75 MHz; $CDCl_3$; Me_4Si) 162.3 (CO), 160.6 (CO), 138.1, 131.0, 129.4, 126.3, 124.1, 121.50, 121.47, 119.3, 118.1 and 102.9 (ArH).

4*H*,6*H*-Isochromeno[5,4-*fg*]quinoxaline-4,6-dione (13)

A mixture of **12** (600 mg, 2.63 mmol) and glyoxal (5.3 cm^3, 46 mmol, 40 wt % solution in water) in H_2O (30 cm^3) was heated at reflux for 24 h. After cooling to room temperature the brown solid was filtered and washed with AcOEt to give **13** (625 mg, 95%) as an unstable brown solid, mp >300 °C; ν_{max}(KBr)/cm^{-1} 1775 and 1750; δ_H (300 MHz; CF_3CO_2D; Me_4Si) 9.98 (1 H, d, *J* 2.7, PyrazineH), 9.93 (1 H, d, *J* 8.7, ArH), 9.54 (1 H, d, *J* 2.7, PyrazineH), 9.53 (1 H, s, ArH), 9.16 (1 H, d, *J* 7.3, ArH) and 8.47 (1 H, m, ArH); δ_C (75 MHz; CF_3CO_2D; Me_4Si) 163.6 (CO), 162.5 (CO), 151.7, 148.3, 141.0, 139.1, 135.3, 133.8, 132.3, 131.1, 129.8, 127.3 and 120.9 (ArH).

9,10-Bis(trifluoromethyl)-4*H*,6*H*-isochromeno[5,4-*fg*]quinoxaline-4,6-dione (14)

To a suspension of compound **12** (1.0 g, 4.39 mmol) in DMF (7 cm^3) under argon, was added dropwise 1,1,1,4,4,4-hexafluorobutane-2,3-dione[15] (2.0 g, 10.54 mmol) in DMF (10 cm^3) at 0 °C. The mixture was stirred at room temperature overnight, and then the solvent was removed under reduced pressure at room temperature. The residue was purified by flash chromatography using hexane–AcOEt (8 : 2) as eluent to give **14** (339 mg, 20%) as a white solid, mp 197–200 °C (from AcOEt–hexane) (Found: C, 49.9; H, 1.1; N, 7.3. $C_{16}H_4F_6N_2O_3$ requires C, 49.8; H, 1.0; N, 7.25%); ν_{max}(KBr)/cm^{-1} 1780 and 1755; δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.67 (1 H, dd, *J* 8.2 and 1.1, ArH), 9.30 (1 H, s, ArH), 8.95 (1 H, dd, *J* 7.7 and 1.1, ArH) and 8.26 (1 H, m, ArH); δ_C (75 MHz; $CDCl_3$; Me_4Si) 159.9 (CO), 159.5 (CO), 141.3, 140.2, 134.3, 131.9, 131.6, 131.4, 131.1, 130.6, 130.4, 128.1, 126.1, 120.7 (ArH) and 120.2 (q, $^1J_{C,F}$ 275, 2 × CF_3); *m*/*z* (EI) 386 (M^+, 59%), 342 (100), 314 (48) and 152 (28).

General procedure for the preparation of mononaphthalimides and their salts

A suspension of corresponding anhydride in toluene was treated with an excess of the corresponding polyamine in absolute EtOH. The mixture was heated at reflux temperature until the reaction was completed (TLC). The precipitated solid was filtered and recrystallized from the appropriate solvent to provide the mononaphthalimide as a free base. Compounds **1** and **2** were suspended in absolute EtOH and methanesulfonic acid (2.2 eq.) was added. The monoimide salts were isolated by filtration and washed with diethyl ether.

5,6-Diamino-2-[2-(dimethylamino)ethyl]-1*H*-benz[*de*]isoquinoline-1,3(2*H*)-dione (7)

From **12** (200 mg, 0.88 mmol) in toluene (5 cm^3) and *N,N*-dimethylethane-1,2-diamine (387 mg, 4.4 mmol) in absolute EtOH (3 cm^3) yielded **7** (145 mg, 55%) as a red solid, mp 220–227 °C (from absolute EtOH) (Found: C, 63.9; H, 6.0; N, 18.0. $C_{16}H_{18}N_4O_2$ requires C, 64.0; H, 6.1; N, 18.25%); ν_{max}(KBr)/cm^{-1} 3440, 3380, 3290, 3230, 1680 and 1630; δ_H (300 MHz; DMSO-d_6; Me_4Si) 8.48 (1 H, d, *J* 8.5, ArH), 8.19 (1 H, d, *J* 7.3, ArH), 7.92 (1 H, s, ArH), 7.54 (1 H, m, ArH), 6.46 (2 H, br s, NH_2), 5.12 (2 H, br s, NH_2), 4.12 (2 H, t, *J* 6.7, CH_2NCO), 2.47 (2 H, t, *J* 6.7, CH_2N) and 2.20 (6 H, s, 2 × CH_3); δ_C (75 MHz; DMSO-d_6; Me_4Si) 163.9 (CO), 163.0 (CO), 136.3, 130.4, 127.9, 127.1, 123.6, 123.2, 121.6, 120.8, 119.5, 108.3 (ArH), 56.6 (CH_2), 45.3 (CH_3) and 37.1 (CH_2); *m*/*z* (ESI) 299 $[M + H]^+$.

5-[2-(Dimethylamino)ethyl]-4*H*-isoquino[6,5,4-*fg*]quinoxaline-4,6(5*H*)-dione (1)

From **13** (125 mg, 0.50 mmol) in toluene (4 cm^3) and *N,N*-dimethylethane-1,2-diamine (80 mg, 0.91 mmol) in absolute EtOH (2 cm^3) yielded **1** (95 mg, 59%) as a brown solid, mp 149–151 °C (from absolute EtOH–toluene); ν_{max}(KBr)/cm^{-1} 1700 and 1655; δ_H (300 MHz; DMSO-d_6; Me_4Si) 9.28 (1 H, d, *J* 7.3, ArH), 9.20 (2 H, m, PyrazineH), 8.67 (1 H, s, ArH), 8.57 (1 H, d, *J* 7.3, ArH), 8.05 (1 H, m, ArH), 4.16 (2 H, t, *J* 6.7, CH_2NCO), 2.54 (2 H, t, *J* 6.7, CH_2N) and 2.24 (6 H, s, 2 × CH_3). The free base was converted into the corresponding methanesulfonate sesquihydrate (88%), mp 217–220 °C (from absolute EtOH) (Found: C, 51.1; H, 5.0; N, 12.4; S, 7.3. $C_{18}H_{16}N_4O_2{\cdot}CH_3SO_3H{\cdot}1.5H_2O$ requires C, 51.4; H, 5.2; N, 12.6; S, 7.5%); ν_{max}(KBr)/cm^{-1} 2650, 1700 and 1660; δ_H (300 MHz; D_2O; Me_4Si) 8.72 (2 H, br s, PyrazineH), 8.48 (1 H, d, *J* 8.3, ArH), 8.05 (1 H, d, *J* 7.7, ArH), 7.89 (1 H, s, ArH), 7.51 (1 H, dd, *J* 8.3 and 7.7, ArH), 4.20 (2 H, t, *J* 6.0, CH_2NCO), 3.27 (2 H, t, *J* 6.0, CH_2N), 2.80 (6 H, s, 2 × CH_3N) and 2.52 (3 H, s, $CH_3SO_3^-$); δ_C (300 MHz; D_2O; Me_4Si) 164.9 (CO), 164.1 (CO), 148.2, 147.6, 142.3, 139.9, 132.9, 132.8, 130.9, 129.9, 128.8, 126.6, 123.4, 121.4 (ArH), 55.9 (CH_2), 44.3 (CH_3), 39.4 ($CH_3SO_3^-$) and 36.4 (CH_2).

5-[2-(Dimethylamino)ethyl]-9,10-bis(trifluoromethyl)-4*H*-isoquino[6,5,4-*fg*]quinoxaline-4,6(5*H*)-dione (2)

From **14** (150 mg, 0.39 mmol) in toluene (3 cm^3) and *N,N*-dimethylethane-1,2-diamine (103 mg, 1.17 mmol) in absolute EtOH (1 cm^3) yielded **2** (128 mg, 72%) as a yellow solid, mp 119–121 °C (from cyclohexane); ν_{max}(KBr)/cm^{-1} 1715 and 1670; δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.54 (1 H, dd, *J* 7.1 and 1.1, ArH), 9.23 (1 H, s, ArH), 8.90 (1 H, dd, *J* 7.1 and 1.1, ArH), 8.16 (1 H, m, ArH), 4.40 (2 H, dd, *J* 7.1 and 6.6, CH_2NCO), 2.71 (2 H, dd, *J* 7.1 and 6.6, CH_2N) and 2.37 (6 H, s, 2 × CH_3). The free base was converted into the corresponding methanesulfonate monohydrate (88%), mp 142 °C (dec) (Found: C, 44.5; H, 3.3; N, 10.0; S, 5.55. $C_{20}H_{14}F_6N_4O_2{\cdot}CH_3SO_3H{\cdot}H_2O$ requires C, 44.2; H, 3.5; N, 9.8; S, 5.6%); ν_{max}(KBr)/cm^{-1} 2640, 1710 and 1670; δ_H (300 MHz; DMSO-d_6; Me_4Si) 9.49 (1 H, d, *J* 7.9, ArH), 9.21 (1 H, br s, NH^+), 8.99 (1 H, s, ArH), 8.86 (1 H, d, *J* 6.7, ArH), 8.33 (1 H, m, ArH), 4.47 (2 H, t, *J* 5.5, CH_2NCO), 3.52 (2 H, m, CH_2N), 2.96 (3 H, s, CH_3N), 2.94 (3 H, s, CH_3N) and 2.34 (3 H, s, $CH_3SO_3^-$); δ_C (75 MHz; DMSO-d_6; Me_4Si) 163.4 (CO), 162.7 (CO), 141.4, 140.4, 133.0, 130.7, 130.4, 130.3, 128.5, 128.4, 128.0, 123.3 (ArH), 120.2 (q, $^1J_{C,F}$ 275, 2 × CF_3), 54.7 (CH_2), 42.9 (CH_3), 39.6 ($CH_3SO_3^-$) and 35.5 (CH_2); *m*/*z* (ESI) 457 $[M + H]^+$.

General procedure for the preparation of bisnaphthalimides and their salts

A suspension of corresponding anhydride in toluene was treated with the corresponding polyamine in absolute EtOH. The mixture was heated at reflux temperature until the reaction was completed (TLC). The precipitated solid was filtered and recrystallized from the appropriate solvent to provide the bisnaphthalimide as a free base. Compounds **3**–**6** were suspended in absolute EtOH and methanesulfonic acid (2.5 eq.) was added. The bisimide salts were isolated by filtration and washed with diethyl ether.

***N,N′*-Bis[2-(5,6-diamino-1,3-dioxo-2,3-dihydro-1*H*-benz[*de*]-isoquinolin-2-yl)ethyl]propane-1,3-diamine (8)**

To a hot suspension of compound **12** (137 mg, 0.60 mmol) in toluene (3 cm^3), was added dropwise *N,N′*-bis(2-aminoethyl)propane-1,3-diamine (100 mg, 0.62 mmol). The reaction mixture was refluxed for 4 days, and then the precipitated solid was filtered to give **8** (133 mg, 76%) as a red solid, mp 177 °C (dec) (from toluene) (Found: C, 60.4; H, 5.55; N, 17.8. $C_{31}H_{32}N_8O_4{\cdot}2H_2O$ requires C, 60.4; H, 5.8; N, 18.1%); ν_{max}(KBr)/cm^{-1} 3370, 3300, 3240, 1675 and 1630; δ_H (300 MHz; DMSO-d_6; Me_4Si) 8.48 (2 H, d, *J* 8.6, ArH), 8.19 (2 H, d, *J* 6.7, ArH), 7.92 (2 H, s, ArH), 7.53 (2 H, m, ArH), 6.51 (4 H, br s, 2 × NH_2), 5.16 (4 H, br s, 2 × NH_2), 4.07 (4 H, t, *J* 6.7, 2 × CH_2NCO), 2.72 (4 H, t, *J* 6.7, 2 × CH_2N), 2.57 (4 H, t, *J* 6.7, 2 × CH_2N) and 1.50 (2 H, m, CH_2); δ_C (75 MHz; DMSO-d_6; Me_4Si) 164.1 (CO), 163.2 (CO), 136.3, 130.4, 127.8, 127.1, 123.6, 123.3, 121.7, 120.8, 119.6, 108.4 (ArH), 47.1 (CH_2N), 47.0 (CH_2N), 38.8 (CH_2N) and 29.4 (CH_2); *m*/*z* (ESI) 581 $[M + H]^+$.

***N,N′*-Bis[2-(5,6-diamino-1,3-dioxo-2,3-dihydro-1*H*-benz[*de*]-isoquinolin-2-yl)ethyl]-*N,N′*-dimethylpropane-1,3-diamine (9)**

To a hot suspension of compound **12** (200 mg, 0.88 mmol) in toluene (5 cm^3), was added dropwise *N,N′*-bis(2-aminoethyl)-*N,N′*-dimethylpropane-1,3-diamine (83 mg, 0.44 mmol). After the mixture was refluxed for 5 days, an excess of amine (109 mg, 0.58 mmol) was added. The reaction mixture was refluxed for 7 days, and then the precipitated solid was filtered and washed with AcOEt and hexane to give **9** (220 mg, 82%) as a red solid, mp 200 °C (dec) (Found: C, 56.5; H, 6.4; N, 16.4. $C_{33}H_{36}N_8O_4{\cdot}5H_2O$ requires C, 56.7; H, 6.6; N, 16.1%); ν_{max}(KBr)/cm^{-1} 3420, 3320, 3250, 1705 and 1660; δ_H (300 MHz; DMSO-d_6; Me_4Si) 8.46 (2 H, d, *J* 8.5, ArH), 8.16 (2 H, d, *J* 7.0, ArH), 7.90 (2 H, s, ArH), 7.50 (2 H, dd, *J* 8.5 and 7.0, ArH), 6.50 (4 H, br s, 2 × NH_2), 5.15 (4 H, br s, 2 × NH_2), 4.05 (4 H, m, 2 × CH_2NCO), 2.45 (4 H, m, 2 × CH_2N), 2.28 (4 H, m, 2 × CH_2N), 2.13 (6 H, s, 2 × CH_3) and 1.44 (2 H, m, CH_2); δ_C (75 MHz; DMSO-d_6; Me_4Si) 163.9 (CO), 163.0 (CO), 136.2, 130.4, 127.8, 127.0, 123.5, 123.2, 121.6, 120.8, 119.5, 108.4 (ArH), 55.0 (CH_2N), 54.2 (CH_2N), 42.1 (CH_3), 36.8 (CH_2N) and 24.6 (CH_2); *m*/*z* (ESI) 609 $[M + H]^+$.

***N,N′*-Bis[2-(4,6-dioxo-5,6-dihydro-4*H*-isoquino[6,5,4-*fg*]-quinoxalin-5-yl)ethyl]propane-1,3-diamine (3)**

From **13** (250 mg, 1.00 mmol) in toluene (5 cm^3) and *N,N′*-bis(2-aminoethyl)propane-1,3-diamine (80 mg, 0.50 mmol) in absolute EtOH (2 cm^3) yielded **3** (222 mg, 71%) as a yellow solid, mp 195–197 °C (from absolute EtOH–toluene); ν_{max}(KBr)/cm^{-1} 3400, 1700 and 1660; δ_H (300 MHz; DMSO-d_6–CF_3CO_2D; Me_4Si) 9.50 (2 H, d, *J* 8.5, ArH), 9.30 (2 H, d, *J* 1.8, PyrazineH), 9.27 (2 H, d, *J* 1.8, PyrazineH), 8.90 (2 H, s, ArH), 8.72 (2 H, d, *J* 7.3, ArH), 8.18 (2 H, m, ArH), 4.44 (4 H, t, *J* 7.3, 2 × CH_2NCO), 3.38 (4 H, br s, 2 × CH_2N), 3.09 (4 H, t, *J* 7.3, 2 × CH_2N) and 1.95 (2 H, m, CH_2). The free base was converted into the corresponding dimethanesulfonate trihydrate (97%), mp >300 °C (Found: C, 50.8; H, 4.8; N, 12.5; S, 7.6. $C_{35}H_{28}N_8O_4{\cdot}2CH_3SO_3H{\cdot}3H_2O$ requires C, 50.9; H, 5.1; N, 12.8; S, 7.35%); ν_{max}(KBr)/cm^{-1} 3400, 2260, 1700 and 1655; δ_H (300 MHz; D_2O; Me_4Si) 8.41 (4 H, br s, PyrazineH), 7.94 (2 H, d, *J* 8.3, ArH), 7.81 (2 H, d, *J* 7.0, ArH), 7.40 (2 H, s, ArH), 7.20 (2 H, dd, *J* 8.3 and 7.0, ArH), 4.06 (4 H, dd, *J* 6.0 and 5.5, 2 × CH_2NCO), 3.22 (4 H, dd, *J* 6.0 and 5.5, 2 × CH_2N), 3.15 (4 H, dd, *J* 7.7 and 7.1, 2 × CH_2N), 2.52 (6 H, s, 2 × CH_3) and 2.03 (2 H, m, CH_2); δ_C (300 MHz; D_2O; Me_4Si) 164.7 (CO), 163.9 (CO), 148.0, 147.5, 141.5, 139.1, 132.8, 132.0, 130.5, 130.0, 128.1, 126.0, 123.0, 121.1 (ArH), 46.0 (CH_2N), 45.0 (CH_2N), 39.4 (CH_3), 37.5 (CH_2N) and 22.2 (CH_2); *m*/*z* (ESI) 625 $[M + H]^+$.

***N,N′*-Bis[2-(4,6-dioxo-5,6-dihydro-4*H*-isoquino[6,5,4-*fg*]-quinoxalin-5-yl)ethyl]-*N,N′*-dimethylpropane-1,3-diamine (4)**

From **13** (309 mg, 1.24 mmol) in toluene (5 cm^3) and *N,N′*-bis(2-aminoethyl)-*N,N′*-dimethylpropane-1,3-diamine (116 mg, 0.62 mmol) in absolute EtOH (2 cm^3) yielded **4** (395 mg, 98%) as a yellow solid, mp 140 °C (dec) (from absolute EtOH–Et_2O); ν_{max}(KBr)/cm^{-1} 1710 and 1650; δ_H (300 MHz; DMSO-d_6; Me_4Si) 9.09 (2 H, d, *J* 7.9, ArH), 9.03 (2 H, d, *J* 1.8, PyrazineH), 8.94 (2 H, d, *J* 1.8, PyrazineH), 8.54 (2 H, s, ArH), 8.49 (2 H, d, *J* 7.9, ArH), 7.98 (2 H, t, *J* 7.9, ArH), 3.93 (4 H, t, *J* 6.7, 2 × CH_2NCO), 2.46 (4 H, dd, *J* 6.7 and 6.1, 2 × CH_2N), 2.32 (4 H, t, *J* 6.7, 2 × CH_2N), 2.17 (6 H, s, 2 × CH_3) and 1.50 (2 H, m, CH_2). The free base was converted into the corresponding dimethanesulfonate hydrate (77%), mp 192 °C (dec) (Found: C, 55.0; H, 4.9; N, 13.0. $C_{37}H_{32}N_8O_4{\cdot}2CH_3SO_3H{\cdot}H_2O$ requires C, 55.4; H, 4.8; N, 13.3%); ν_{max}(KBr)/cm^{-1} 2640, 1710 and 1660; δ_H (300 MHz; D_2O; Me_4Si) 8.55 (4 H, br s, PyrazineH), 8.11 (2 H, d, *J* 8.2, ArH), 8.01 (2 H, d, *J* 7.1, ArH), 7.67 (2 H, s, ArH), 7.34 (2 H, m, ArH), 4.26 (4 H, m, 2 × CH_2NCO), 3.44 (8 H, m, 4 × CH_2N), 2.95 (6 H, s, 2 × CH_3N), 2.59 (6 H, s, 2 × $CH_3SO_3^-$) and 2.28 (2 H, br s, CH_2); δ_C (300 MHz; D_2O; Me_4Si) 164.3 (CO), 163.7 (CO), 148.1, 147.6, 141.5, 139.1, 132.7, 132.0, 130.5, 129.9, 128.0, 125.8, 122.8, 120.9 (ArH), 53.7 (CH_2N), 52.4 (CH_2N), 42.5 (CH_3), 39.5 ($CH_3SO_3^-$), 35.9 (CH_2N) and 19.1 (CH_2).

***N,N′*-Bis{2-[4,6-dioxo-9,10-bis(trifluoromethyl)-5,6-dihydro-4*H*-isoquino[6,5,4-*fg*]quinoxalin-5-yl]ethyl}propane-1,3-diamine (5)**

To a suspension of compound **14** (197 mg, 0.51 mmol) in toluene (5 cm^3), was added dropwise *N,N′*-bis(2-aminoethyl)-propane-1,3-diamine (41 mg, 0.25 mmol) in absolute EtOH (2 cm^3). The reaction mixture was refluxed for 2 days, and then the solvent was evaporated. The residue was purified by flash chromatography using CH_2Cl_2–MeOH (9 : 1) as eluent to give **5** (146 mg, 64%) as a brown solid; ν_{max}(KBr)/cm^{-1} 3400, 1710 and 1670; δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.43 (2 H, d, *J* 8.3, ArH), 9.09 (2 H, s, ArH), 8.80 (2 H, d, *J* 7.3, ArH), 8.10 (2 H, dd, *J* 8.3 and 7.3, ArH), 4.33 (4 H, t, *J* 6.0, 2 × CH_2NCO), 3.61 (2 H, br s, 2 × NH), 3.11 (4 H, t, *J* 6.0, 2 × CH_2N), 2.92 (4H, t, *J* 6.0, 2 × CH_2N) and 1.83 (2 H, m, CH_2). The free base was converted into the corresponding dimethanesulfonate pentahydrate (77%), mp 113 °C (dec) (Found: C, 41.7; H, 3.6; N, 9.3; S, 5.4. $C_{39}H_{24}F_{12}N_8O_4{\cdot}2CH_3SO_3H{\cdot}5H_2O$ requires C, 41.4; H, 3.5; N, 9.05; S, 5.2%); ν_{max}(KBr)/cm^{-1} 3400, 2800, 1710 and 1670; δ_H (300 MHz; DMSO-d_6; Me_4Si) 9.48 (2 H, d, *J* 7.9, ArH), 8.99 (2 H, s, ArH), 8.85 (2 H, d, *J* 7.9, ArH), 8.70 (4 H, br s, 2 × NH_2^+), 8.32 (2 H, t, *J* 7.9, ArH), 4.44 (4 H, br s, 2 × CH_2NCO), 3.38 (4 H, br s, 2 × CH_2N), 3.11 (4 H, br s, 2 × CH_2N), 2.30 (6 H, s, 2 × CH_3) and 1.96 (2 H, br s, CH_2); δ_C (75 MHz; DMSO-d_6; Me_4Si) 163.4 (CO), 162.7 (CO), 141.3, 140.3, 132.9, 130.6, 130.4, 130.3, 130.2, 128.5, 128.4, 128.0, 123.3 (ArH), 120.2 (q, $^1J_{C,F}$ 274, 2 × CF_3), 44.9 (CH_2N), 44.2 (CH_2N), 39.4 ($CH_3SO_3^-$), 36.7 (CH_2N) and 22.0 (CH_2); *m*/*z* (ESI) 897 $[M + H]^+$.

***N,N′*-Bis{2-[4,6-dioxo-9,10-bis(trifluoromethyl)-5,6-dihydro-4*H*-isoquino[6,5,4-*fg*]quinoxalin-5-yl]ethyl}-*N,N′*-dimethylpropane-1,3-diamine (6)**

To a suspension of compound **14** (124 mg, 0.32 mmol) in toluene (5 cm^3), was added dropwise *N,N′*-bis(2-aminoethyl)-*N,N′*-dimethylpropane-1,3-diamine (30 mg, 0.16 mmol) in absolute EtOH (2 cm^3). The reaction mixture was refluxed for 7 hours, and then the solvent was evaporated. The residue was purified by flash chromatography using CH_2Cl_2–MeOH (95 : 5) as eluent to give **6** (91 mg, 61%) as a brown solid, mp 175–180 °C; ν_{max}(KBr)/cm^{-1} 1710 and 1670; δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.44 (2 H, dd, *J* 8.2 and 1.1, ArH), 9.08 (2 H, s, ArH), 8.81 (2 H, dd, *J* 7.3 and 1.1, ArH), 8.11 (2 H, dd, *J* 8.2 and 7.3, ArH), 4.27 (4 H, dd, *J* 7.1 and 6.6, 2 × CH_2NCO), 2.68 (4 H, dd, *J* 7.1 and 6.6, 2 × CH_2N), 2.45 (4 H, t, *J* 7.1, 2 × CH_2N), 2.31 (6 H, s, 2 × CH_3) and 1.61 (2 H, m, CH_2). The free base was converted into the corresponding dimethanesulfonate (89%), mp 203–206 °C (from absolute EtOH) (Found: C, 42.0; H, 3.7; N, 8.8. $C_{41}H_{28}F_{12}N_8O_4 \cdot 2CH_3SO_3H$ requires C, 42.2; H, 3.95; N, 9.15%); ν_{max}(KBr)/cm^{-1} 2600, 1710 and 1670; δ_H (300 MHz; MeOD-d_4; Me_4Si) 9.43 (2 H, d, *J* 7.9, ArH), 9.02 (2 H, s, ArH), 8.83 (2 H, d, *J* 7.9, ArH), 8.17 (2 H, t, *J* 7.9, ArH), 4.67 (4 H, dd, *J* 6.1 and 5.5, 2 × CH_2NCO), 3.68 (6 H, br s, 3 × CH_2N), 3.53 (2 H, br s, CH_2N), 3.12 (6 H, s, 2 × CH_3N) and 2.37 (8 H, br s, 2 × $CH_3SO_3^-$, CH_2); δ_C (75 MHz; DMSO-d_6; Me_4Si) 163.4 (CO), 162.8 (CO), 141.3, 140.3, 133.1, 130.7, 130.50, 130.49, 128.6, 128.5, 128.0, 123.3 (ArH), 120.3 (q, $^1J_{C,F}$ 274, 2 × CF_3), 52.7 (CH_2N), 52.0 (CH_2N), 40.2 (CH_3), 39.6 ($CH_3SO_3^-$), 35.2 (CH_2N) and 18.0 (CH_2); *m/z* (ESI) 925 $[M + H]^+$.

In vitro cytotoxicity assays

The cell lines used were: human colon carcinoma (HT-29) (ATCC, HTB 38), human cervical carcinoma (HeLa) (ATCC, CCL 2) and human prostate carcinoma (PC-3) (ECACC, 90112714). For each experiment, cultures were seeded from frozen stocks. Each cell line was maintained in its appropriated medium and was incubated at 37 °C in a 5% CO_2 atmosphere.

All cell lines were in the logarithmic phase of growth when the assay of MTT was carried out. Cells were harvested and seeded into 96-well tissue culture plates at a density of 2.5×10^3 cells/well in 150 μL aliquots of medium. The concentrations tested were serial dilutions of a stock solution (1×10^{-5} M in DMSO) with phosphate-buffered saline (PBS) and were added 24 h later. The assay was ended after 72 h of drug exposure and PBS was used as a negative control and doxorubicine as a positive control.

After a 72 h exposure period, cells were washed twice with PBS, and then 50 μL/well of MTT reagent (1 mg cm^{-3} in PBS; Sigma) and 150 μL/well of prewarmed medium were added. The plates were returned to the incubator for 4 h. Subsequently, DMSO was added as solvent. Absorbance was determined at 570 nm with a Microplate reader (Opsys MR).

All experiments were performed at least three times, and the average of the percentage absorbance was plotted against concentration. Then, the concentration of drug required to inhibit 50% of cell growth (IC_{50}) was calculated for each compound.

DNA binding experiments

DNA

Calf thymus DNA was purchased from Sigma Chemical Co. as the highly polymerized sodium salt. For viscometric experiments the DNA was sonicated to fragments of approx. 4.5×10^5 D determined as described by Eigner and Doty.[16] The sonicated DNA sample displayed an A_{260} : A_{280} ratio of 1.92. This spectral data is consistent with published values.[17]

Viscometric titrations

The viscometric measurements were performed in an Ubbelohde Microviscometer at 25 ± 0.05 °C. Solutions of sonicated DNA and the selected compound were prepared in tris buffer (50 mM, pH = 6.9). These solutions had different molar ratio, *r*, of added compound to DNA nucleotides. Flow rates were measured with a Schott-Geräte Viscosystem AVS 350 to an accuracy of 0.01%. Time readings were recorded in triplicate to 0.01 s.

UV–VIS

For spectrometric determinations a UV-1603 Shimadzu was used. The spectra of sonicated DNA, intercalator and DNA–intercalator complex were registered at different temperatures: 5, 10, 25 and 35 °C.

Melting temperature (*T*m) measurements

Melting curves were obtained in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) with a heating rate of 1 °C min^{-1}. The "melting" temperature *T*m was taken as the mid-point of the hyperchromic transition.

Alkaline single-cell gel electrophoresis assay

The alkaline single-cell gel electrophoresis assay (comet assay) detects DNA damage in individual cells embedded in agarose. The test was performed on HT-29 cells following the method described by Moinet-Hedin *et al.*[12] After 1 h of treatment with the drug, cells were centrifuged and resuspended in low-melting-point (LMP) agarose at 37 °C. The cell suspension was put on a slide precoated with normal agarose and a glass cover slip was added. After solidification at 0 °C the glass cover slip was gently removed and a third layer of 0.5% of LMP agarose in PBS was added and run for solidification.

The slides were put in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM tris, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) for 1 h and were rinsed in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 40 min. Electrophoresis (300 mA, 0.7 V cm^{-1}) was then performed for 24 min in fresh buffer. The slides were washed twice in neutralization buffer (0.4 M tris, pH 7.5) and stained with ethidium bromide (20 μg cm^{-3}). They were observed using a fluorescence microscope (Nikon) with an excitation filter of 515–560 nm and a barrier filter of 580 nm.

DNA *topoisomerase* inhibition

For the DNA relaxation experiments, supercoiled pKMp27 DNA (0.5 μg) was incubated with 4 units human *topoisomerase I* or *II*. (TopoGen Inc.) at 37 °C for 1 h in relaxation buffer (50 mM tris pH 7.8, 59 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the tested drug. Reactions were terminated by adding SDS (dodecyl sulfate sodium salt) to 0.25% and *proteinase K* to 250 μg cm^{-3}. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in an ethidium-containing 1% agarose gel for 2 h at 120 V. Gels were washed and photographed under UV light.[18]

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