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Original article

Synthesis and antitumoral activity of novel thiazolobenzotriazole, thiazoloindolo[3,2-*c*]quinoline and quinolinoquinoline derivatives

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ABSTRACT

The biological evaluation of some novel thiazoloindolo[3,2-*c*]quinoline, 8-substituted-11*H*-indolo[3,2-*c*]quinolines is described. These compounds were obtained *via* Graebe–Ullmann thermal cyclization from appropriated *N*-arylated benzotriazoles. 7*H*-4,7-Diaza-benzo[*de*]anthracene, a reaction by-product structurally closed to the pyridoacridine skeleton was also identified. All thiazolobenzotriazole intermediates were tested *in vitro* for their capacity to inhibit the growth of two breast cancer cell lines, MCF-7 and MDA-MB-231. In parallel, the newly synthesized skeletons were evaluated for DNA interaction, topoisomerases' inhibition, and cytotoxicity against HL60 and HL60/MX2 human leukemia cells. Most compounds showed a potent growth inhibitory effect on all the tested cell lines, with IC_{50} in the μM range.

1. Introduction

Numerous indoloquinoline alkaloids have been identified from extracts of the West African plant *Cryptolepis sanguinolenta*, such as isocryptolepine **I**, also referred to as cryptosanguinolentine (Fig. 1). The indolo[3,2-*c*]quinoline (benzo-γ-carboline) [1,2] structure is rare in nature and generates a large interest of academic and industrial research groups [3–12].

Owing to their growing use as compounds of therapeutic importance (antibacterial, antiplasmodial and anticancer drugs) [3–12], the synthesis of indoloquinoline derivatives has been actively pursued in the past ten years. Cryptolepine, the lead alkaloid of this family, is a potent DNA-binding cytotoxic agent interfering with topoisomerase II, which inhibits DNA synthesis in melanoma cells [13].

The thiazole ring, present in various natural and synthetic products, has generated interest of many groups on account of its useful biological properties [14–18]. Our interest in biologically active compounds as potential antitumor agents focussed our studies on the synthesis of new derivatives in which the thiazole ring might be fused to polyheterocyclic systems [19–22]. In this context, we previously reported the synthesis of a novel thiazoloindolo[3,2-*c*]quinoline **II** skeleton as outlined in Scheme 1, besides unexpected rare 10-*N*-substituted-7*H*-4,7-diaza-benzo[*de*]anthracene **III** (Fig. 1) *via* the Graebe–Ullmann thermal ring transformation [23].

N-(7*H*-4,7-Diaza-benzo[*de*]anthracen-10-yl)-acetamide **III** is structurally closed to the pyrodoacridine skeleton found in many marine alkaloids. It can be considered as an interesting intermediate for the preparation of novel rings [7,24]. The biological potential of these new ring systems, **II** and **III**, remains to be established with the use of specific molecules. These considerations prompted us to examine their ability to interact with DNA, to inhibit with topoisomerases and their anti-proliferative activity, in the hope to find a new lead compound.

Along these lines, we decided to prepare, from appropriated thiazolobenzotriazoles, novel series of thiazoloindoloquinoline or thiazolocarbolines **IV–VI** bearing different substituents at position C-2, and also to synthesize the thiazoloindolo[3,2-*c*]isoquinoline

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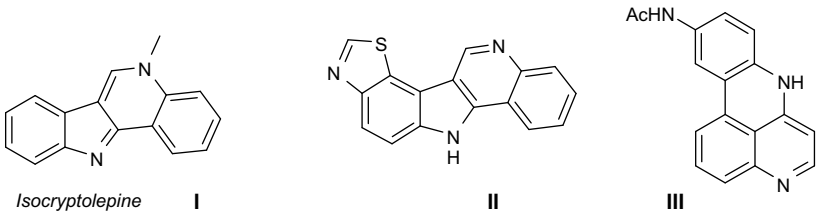


Fig. 1. Structures of a terrestrial natural indolo[3,2-*c*]quinoline (**I**) and synthetic heterocyclic skeletons (**II** and **III**).

analogue **VII**, in which only one nitrogen atom position has been shifted compared to **II** (Fig. 2).

We report here the synthesis, biochemical and biological activities of new indoloquinoline and thiazolobenzotriazole derivatives designed as potential anticancer agents. Our study shed light on their capacity to bind to DNA and to interfere with human topoisomerases, along with their *in vitro* anti-proliferative activity on human breast cancer and leukemia cell lines.

2. Results and discussion

2.1. Chemistry

In continuation with our research program on biologically active fused thiazolo derivatives, we prepared a series of regioisomers of *N*-arylated cyanobenzotriazoles. Substitution of the nitrogen atom was realized by a quinoline (**6**–**11**) or a pyridine ring (**12** and **13**). The synthesis was performed under microwave irradiation with a dedicated microwave oven using 2-cyanothiazolobenzotriazole **1** as starting synthetic material (Scheme 2, Table 1) [23,25]. In all cases, 1 equiv. of commercially available 4-chloroquinoline **2**, 4-chloro-2-methylquinoline, 2-chloroquinoline **3**, **4** or 4-chloropyridine **5** was mixed to **1** and dissolved with a minimum of toluene. After irradiation, it afforded two condensed isomers in moderate to poor yields (Table 1). The major compound in all cases being the less hindered steric product.

It is known that the cyano group at position 2 of the benzothiazole ring is very reactive. With the aim to enhance the cytotoxic activity of such products, modifications by an amino side-group of key synthetic cyano precursors were studied. According to this strategy, condensation of commercially available ethylenediamine with 2-cyanothiazolobenzotriazoles **6**, **8**, **10**, **12**, in ethanol, led to the desired imidazolines **14**–**17** (Scheme 3).

Whatever the experimental conditions, exposing the obtained thiazolobenzotriazoles (**14**–**17**) to microwave irradiation, neat in sealed vial or in the presence of pyrophosphoric acid was unsuccessful and led to complicated mixture. No trace of expected products **IV**–**VI** was detected.

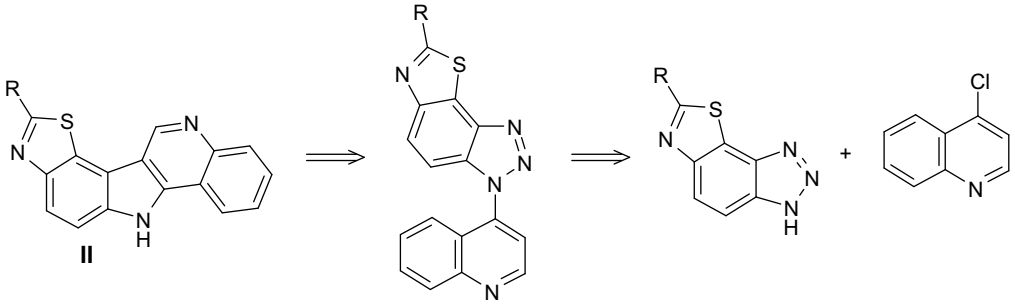
We also tried to synthesize a thiazoloindolo[3,2-*c*]isoquinoline analogue (**VII**), in which only one nitrogen atom has a different position. To the best of our knowledge, the chemical behaviour of 4-isoquinolinyl-functionalized benzotriazoles under thermal cyclization has never been reported. The preparation of isoquinolinylbenzotriazole by condensation of commercially available 4-bromoisoquinoline **18** and the corresponding cyanothiazolobenzotriazole **1** under neat conditions at high temperature led to carbonaceous mixtures (Scheme 4). *N*-Arylation was mediated either by copper or by palladium catalysis. Whatever the experimental conditions, substitution of isoquinolyl bromide failed.

After several unfruitful attempts to obtain functionalized isoquinoline **VII**, we decided to investigate the reaction from the symmetrical 5,6-dimethylbenzotriazole **19** (Scheme 5).

A long exposition at high temperature was unfruitful and led to the degradation of the starting material. Longer reaction time at a lowest temperature yielded mainly unchanged starting material. After several trials with various acids, the reaction was performed under microwave irradiation, neat in glass vial with a screw cap lid, for 2 h. Among all products, we succeeded once to isolate a trace of compound **19**. The ^{1}H NMR spectrum could fit well for a fused quinolinoquinoline structure (9,10-dimethyl-7*H*-5,7-diaza-benzo[-*de*]anthracene) showing an *H*–C4 characteristic singlet and an *H*–C-2 triplet.

2.2. DNA binding and effects on DNA topoisomerases (Fig. 3)

Ring systems **II**, **III** were investigated for DNA binding and topoisomerases' inhibition. 8-Aminoindolo[3,2-*c*]quinoline **VIII** synthesized in our previous work [23] was also evaluated because of its interesting structure, close to the ring formed above. The three compounds bear a planar heterocyclic chromophore susceptible to intercalate between DNA base pairs. The interaction was first monitored by absorption spectroscopy and typical data obtained with compound **III** are presented in Fig. 4. Binding of the molecule to calf thymus DNA (CT DNA contains 42% GC base pairs) causes hypochromic and bathochromic shifts. The molecule absorption band centered at 300 nm strongly decreased upon addition of DNA



Scheme 1. Retrosynthetic pathway for the synthesis of thiazoloindoloquinoline **II**.

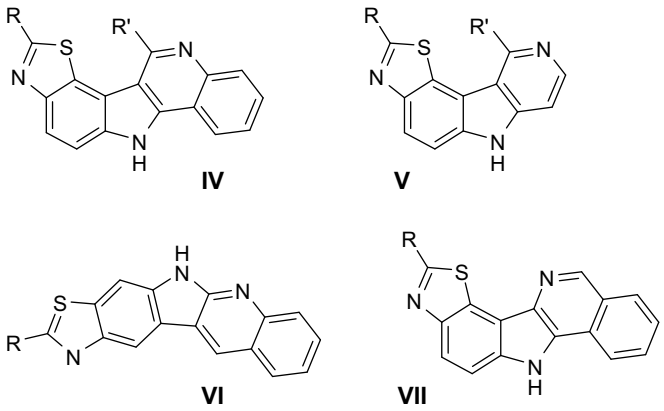


Fig. 2. Structures of **VI** and **VII**.

and a significant red shift occurred. There is no doubt that the molecule forms tight complexes with DNA.

Relative binding affinities for DNA were determined using melting temperature (T_m) experiments. T_m measurements are conventionally used to appreciate the capacity of the molecules to bind to DNA and to stabilize the double helix. The T_m analyses were performed with both CT DNA and the polynucleotide poly(dAT)$_2$ which melted at a lower temperature. The variations of the T_m values ($\Delta T_m = T_m$ drug–DNA complex $- T_m$ DNA alone) are presented in Table 2. The three molecules can strongly bind to DNA and rank in the order **III** > **VIII** > **II**. They can bind to CT DNA and to poly(dAT)$_2$ as well, without any apparent base selectivity. DNase I footprinting experiments (not shown) confirmed that the molecules do not present preferential binding sites (no footprint). They behave as typical DNA-intercalating agents.

Additional evidences of DNA binding come from DNA unwinding experiments. Drug-induced unwinding of a supercoiled plasmid DNA was monitored by a DNA relaxation assay using topoisomerase I [26]. The drug is incubated with the plasmid in the presence of the enzyme and the topoisomers are resolved by electrophoresis. In the absence of ethidium bromide in the agarose gel during the electrophoresis, the relaxation of supercoiled DNA by topoisomerase I leads to a population of topoisomers and the presence of an intercalating agent affects the distribution of the topoisomers' population due to an unwinding effect. A set of data for compounds **III** and **VIII** are presented in Fig. 5A. There is no doubt that these two molecules behave as typical DNA-intercalating agents by producing dose-dependent alterations in plasmid linking. The electrophoresis profile changes markedly in the presence of increasing concentrations of the molecule. In both cases, two phases can be distinguished. First at low concentrations, the intercalation between DNA base pairs induces a relaxation of DNA and the bulky, fully relaxed form migrates slowly through the gel. As the drug concentration increases, the DNA molecules wind in the opposite way so as to produce more compact, positive supercoils which then migrate faster than negatively supercoiled DNA topoisomers. When the DNA is fully positively supercoiled, it migrates as a single band with an electrophoretic mobility close to that of the native negatively supercoiled plasmid (lane "DNA"). This kind of up-and-down profile is typical of an intercalating agent.

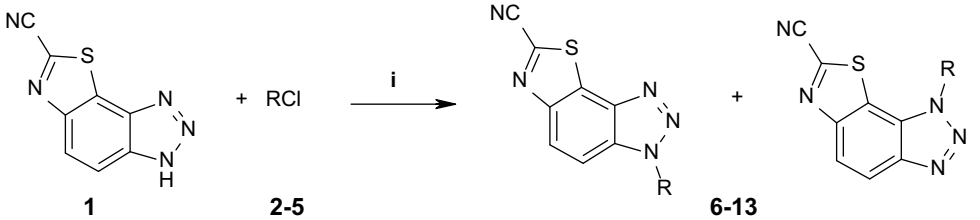
We also investigated topoisomerases' inhibition, using a similar procedure but in the presence of ethidium bromide during the electrophoresis. Poisoning of the enzyme, as with the reference drug camptothecin, leads to single strand breaks, visualized by the appearance of the nicked DNA form. In contrast to camptothecin, the tested molecules do not stabilize topoisomerase I–DNA covalent complex. They do not promote DNA cleavage by topoisomerase I (Fig. 5B) and similar data were obtained with topoisomerase II. Cell experiments also indicated that the molecules do not function as topoisomerase II poisons. The molecules are almost equally cytotoxic to HL60 leukemia cells and HL60/MX2 cells resistant to the reference topoisomerase II poison mitoxantrone. The HL60/MX2 cell line displays an atypical multidrug resistance profile with a decreased expression and activity of topoisomerase II. In other words, the compound cytotoxicity is not linked to topoisomerase II inhibition.

2.3. Effect on the growth of human breast tumor cell lines

The anti-proliferative activity of the thiazolobenzotriazole derivatives was determined using a pair of breast cancer cell lines: MCF-7 and on MDA-MB-231. Results are summarized in Table 3. Most molecules exhibited a moderate-to-strong activity at 10 μM with both cell lines. MDA-MB-231 cells appeared slightly more resistant than MCF-7 cells when exposed to the quinolinotriazoles except with pyridonezotriazoles **10** and **16**. The presence of an imidazoline cationic side chain substituent on the thiazole ring was associated to a stronger cytotoxicity, as described in other cases (**14**–**16**), except for compound **17** bearing a C-2 quinolinyl substituent. The linear aminothiazoloindolo[3,2-*c*]quinoline derivative **VIII**, which acts as a DNA-intercalating agent, also displays a significant anti-proliferative activity.

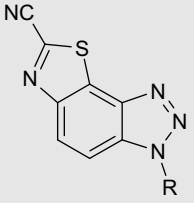
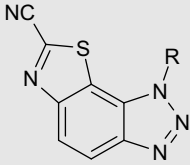
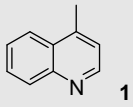
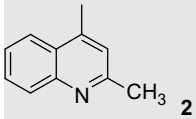
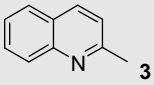
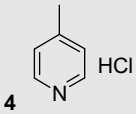
3. Conclusion

In conclusion, we showed that the newly synthesized substituted benzotriazolyl-quinoline derivatives exhibit interesting cytotoxic activity *in vitro* against MCF-7 and MDA-MB-231 tumor cells. Some of them constitute convenient precursor for the synthesis of original fused-ring systems like thiazoloindolo[3,2-*c*]quinolines or quinolinoquinolines. *N*-(7*H*-4,7-Diaza-benzo[-*de*]anthracen-10-yl)-acetamide **III** which is structurally closed to the pyrodoacridine skeleton present in marine alkaloid and thiazoloindolo[3,2-*c*]quinoline **II** could be considered as new lead compounds. These molecules function as DNA-intercalating agents and also display cytotoxic activity against HL60 and HL60/MX2 human leukemia cells. The pharmacological targets of these original heterocycles remain to be established.



Scheme 2. Microwave-assisted synthesis of the 2-cyano-*N*-substituted benzotriazoles **6**–**13** (for R–Cl derivatives and yields see Table 1). (i) Toluene, 160 °C, 1 h (for **6**, **7**); pyridine, 115 °C, 1.5 h (for **8**, **9**), 45 min (for **10**–**13**).

Table 1
Synthesis of the regioisomers of *N*-arylated thiazolobenzotriazoles.

R		
 **1**	**6** 44%	**7** 33%
 **2**	**8** 29%	**9** 21%
 **3**	**10** 15%	**11** 11%
 **4**	**12** 39%	**13** 6%

4. Experimental

4.1. Chemistry

All solvents and reagents were of reagent grade and were used without purification. Melting points were determined using a Köfler melting point apparatus and are not corrected. IR spectra were recorded on a Perkin–Elmer Paragon 1000PC instrument. ^{1}H and ^{13}C NMR were recorded on a JEOL JNM LA400 (400 MHz) spectrometer (Centre Commun d'Analyses, Université de la Rochelle); chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) which was used as internal standard. Coupling constants *J* are given in Hz. The mass spectra (HRMS) were recorded on a Varian MAT311 spectrometer in the Centre Régional de Mesures Physiques de l'Ouest (CRMPO), Université de Rennes. Column chromatography was performed by using Merck silica gel (70–230 mesh) at medium pressure. Light petroleum ether refers to the fraction boiling point 40–60 °C. Analytical thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F254 aluminium packed plates. Focused microwave irradiations were carried out with a CEM Discover™ focused microwave reactor (300 W, 2450 MHz, monomode system), which has in situ magnetic variable speed rotation, irradiation monitored by PC computer, infrared measurement and continuous feedback temperature control. Experiments may be performed at atmospheric pressure or in a sealed tube in pressure-rated reaction tubes with continuous pressure measurement.

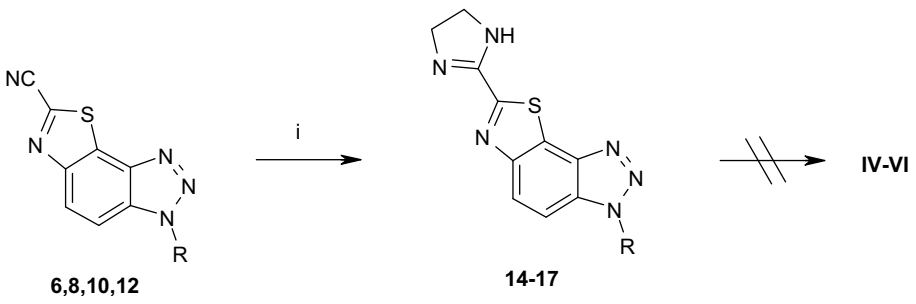
Compounds **1**–**VIII** were prepared according to literature data [23].

4.1.1. Synthesis of quinolinylbenzotriazoles

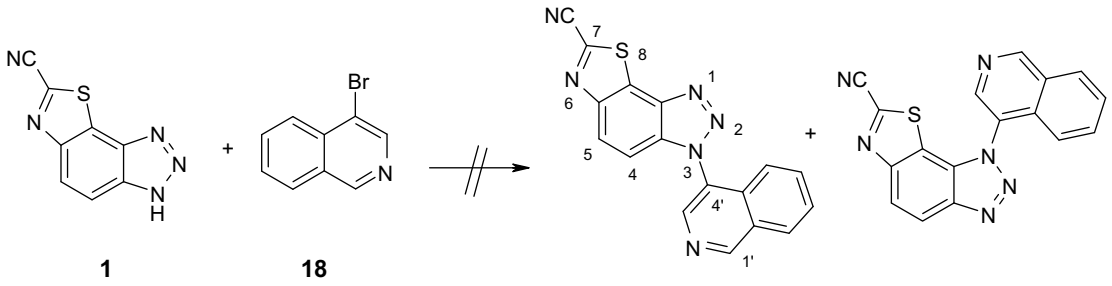
Under an inert atmosphere, a solution of an equimolar mixture of 4-chloroquinoline **2** (0.12 g, 0.73 mmol) and thiazolobenzotriazole **1** (0.14 g, 0.73 mmol) in toluene (2 mL) was heated at 160 °C in a sealed tube for 1 h. After cooling, the toluene was removed under reduced pressure. The mixture was diluted with dichloromethane (15 mL) and washed with water (15 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by column chromatography (silica gel, light petroleum ether/ethyl acetate, 80:20) to provide 2 regioisomers **6** and **7** in 44% and 33% yields respectively.

*4.1.1.1. 3-Quinolin-4-yl-1*H*-thiazolo[4′,5′;3′4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**6**).* Yield: 44%. mp 238–240 °C (ethanol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.23 (d, $J = 4.4$ Hz, 1H, 2′-$H_{quinolin.}$), 8.38 (d, $J = 9.2$ Hz, 1H, ArH), 8.33 (d, $J = 9.2$ Hz, 1H, 5-H or 4-H), 7.92 (td, $J = 2.0$ Hz, 6.8 Hz, 1H, ArH), 7.69 (d, $J = 4.4$ Hz, 1H, 3′-$H_{quinolin.}$), 7.68 (td, $J = 2.0$ Hz, 6.8 Hz, 1H, ArH), 7.66 (d, $J = 9.2$ Hz, 1H, ArH), 7.62 (d, $J = 9.2$ Hz, 1H, 4-H or 5-H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 151.00, 150.42 (2′-C), 150.12, 139.75, 136.24, 135.79, 133.81, 131.12 (7′-C), 130.45, 128.77, 126.71, 125.69, 122.91, 122.55, 117.72 (3′-C), 112.63, 111.09. IR (KBr, cm^{-1}) 2921, 2339, 1435, 1260, 805, 760. HRMS (EI) $[M]^+$ ($C_{17}H_8N_6S$): calcd. 328.0531; found 328.0530.

*4.1.1.2. 1-Quinolin-4-yl-1*H*-thiazolo[4′,5′;3′4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**7**).* Yield: 33%. mp 252–254 °C (ethanol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.16 (d, $J = 4.9$ Hz, 1H, 2′-$H_{quinolin.}$), 8.84 (d, $J = 8.0$ Hz, 1H, 4-H or 5-H), 8.31 (d, $J = 8.0$ Hz, 1H, 5-H or 4-H), 8.26 (d, $J = 9.3$ Hz, 1H, ArH), 8.20 (d, $J = 9.3$ Hz, 1H, ArH), 8.12 (d, $J = 4.9$ Hz, 1H, 3′-$H_{quinolin.}$), 7.91 (t, $J = 7.2$ Hz, 1H, ArH), 7.77 (t, $J = 7.2$ Hz, 1H, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 153.09, 150.24, 150.16, 144.73, 143.09, 139.61, 139.65, 130.55, 130.24, 128.69, 125.12, 124.54, 124.03, 120.72, 119.03, 116.17, 112.70. IR (KBr, cm^{-1}) 3069, 2919, 2230, 1715, 1562, 1501, 1432, 1395, 995, 831, 774. HRMS (EI) $[M]^+$ ($C_{17}H_8N_6S$): calcd. 328.0531; found 328.0530.



Scheme 3. Synthesis of the 2-imidazolino-*N*-substituted benzotriazoles **14**–**17**: (i) Ethylenediamine, ethanol, reflux, 1 h, 61–96%.



Scheme 4. Condensation of thiazolobenzotriazole with 4-bromoisoquinoline.

4.1.2. Synthesis of 3-(2-methylquinolinyl)benzotriazole

Under an inert atmosphere, a solution of an equimolar mixture of 4-chloro-2-methylquinoline **3** (0.31 g, 1.54 mmol) and thiazolobenzotriazole **1** (0.31 g, 1.54 mmol) in pyridine (2 mL) was heated in reflux for 90 min. The mixture was extracted with ethyl acetate (2 × 15 mL) and washed with a saturated solution of NaCl (2 × 15 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by column chromatography (silica gel, dichloromethane:methanol 99:1) to provide 2 regioisomers **8** and **9** in 29% and 21% yields respectively.

*4.1.2.1. 3-(2-Methyl-quinolin-4-yl)-3*H*-thiazolo[4′,5′;3′4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**8**).* Yield: 29%. mp 216–218 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.72 (d, J = 8.8 Hz, 1H, 4-H or 5-H), 8.20 (d, J = 8.4 Hz, 1H, 5′-$H_{quinolin}$), 8.17 (dd, J = 1.2 and 8.4 Hz, 1H, 8′-$H_{quinolin}$), 8.11 (dd, J = 1.2 and 8.8 Hz, 1H, 4-H or 5-H), 7.97 (s, 1H, 3′-$H_{quinolin}$), 7.79 (t, J = 8.4 Hz, 1H, ArH), 7.63 (t, J = 8.4 Hz, 1H, ArH), 2.86 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 159.01, 153.13, 149.15, 144.69, 143.49, 139.54, 136.68, 130.84, 128.85, 127.89, 125.16, 124.47, 123.96, 119.12, 118.96, 116.95, 112.64, 60.36 (CH_3). IR (KBr, cm^{-1}) 3433, 2916, 2231 (CN), 1731, 1604. HRMS (EI) $[M]^+$ ($C_{18}H_{10}N_6S$): calcd. 342.0688; found 342.0662.

*4.1.2.2. 1-(2-Methyl-quinolin-4-yl)-1*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**9**).* Yield: 21%. mp 254–256 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.45 (d, J = 9.0 Hz, 1H, 4-H or 5-H), 8.18 (d, J = 8.5 Hz, 1H, ArH), 7.98 (d, J = 9.0 Hz, 1H, 4-H or 5-H), 7.95 (s, 1H, 3′-$H_{quinolin}$), 7.91 (m, 1H, ArH), 7.61 (dd, J = 5.7 and 8.5 Hz, 1H, ArH), 2.84 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 160.35, 150.76, 149.27, 139.52, 139.26, 134.26, 131.33, 129.43, 128.10, 126.50, 125.75 (6-C), 122.98, 121.50, 119.85 (3′-C), 112.77 (7-H), 25.44 (CH_3). IR (KBr, cm^{-1}) 3729, 3472, 2238 (CN), 1755, 1713, 1384, 1252, 1123, 757. HRMS (EI) $[M]^+$ ($C_{18}H_{10}N_6S$): calcd. 342.06877; $[M - N_2]^+$: 314.06262; found 342.0608.

4.1.3. Synthesis of pyridinylbenzotriazole

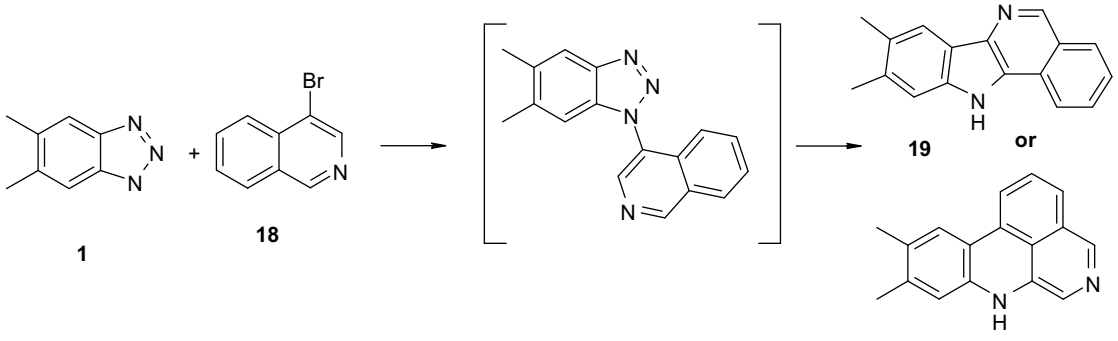
Under an inert atmosphere, a solution of an equimolar mixture of 4-chloropyridine **4** (0.104 g, 0.7 mmol) and thiazolobenzotriazole **1** (0.14 g, 0.7 mmol) in pyridine (2 mL) was heated in reflux for 45 min. The mixture was extracted with dichloromethane (15 mL) and washed water (3 × 10 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by column chromatography (silica gel, dichloromethane:methanol 97:3) to provide 2 regioisomers **10** and **11** in 39% and 6% yields respectively.

*4.1.3.1. 3-Pyridin-4-yl-3*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**10**).* Yield: 39%. mp 244–246 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.88 (dd, J = 1.6 and 5.0 Hz, 2H, 2′-H and 6′-H), 8.29 (dd, J = 1.6 and 5.0 Hz, 2H, 3′-H and 5′-H), 8.20 (d, J = 9.2 Hz, 1H, 4-H or 5-H), 8.10 (d, J = 9.2 Hz, 1H, 4-H or 5-H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 153.31, 151.70, 145.84, 144.86, 139.76, 136.76, 125.33, 124.55, 118.97, 114.15, 112.61, 104.18, 29.69. IR (KBr , cm^{-1}) 3461, 3055, 2233 (CN), 1719, 1586, 1297. HRMS (EI) $[M]^+$ ($C_{13}H_6N_6S$): calcd. 278.0375; found 278.0379.

*4.1.3.2. 1-Pyridin-4-yl-1*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**11**).* Yield: 6%. mp 222–224 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.97 (dd, J = 5.6 and 1.2 Hz, 2H, 2′-H and 6′-H), 8.41 (d, J = 9.2 Hz, 1H, 4-H or 5-H), 8.08 (d, J = 9.2 Hz, 1H, 4-H or 5-H), 7.88 (dd, J = 5.6 and 1.2 Hz, 2H, 3′-H and 5′-H). IR (KBr, cm^{-1}) 3568, 3080, 2239 (CN), 1733, 1596, 1410, 1203. HRMS (EI) $[M - CN]^+$ ($C_{12}H_6N_5S$): calcd. 252.0344; found 252.0355.

4.1.4. Synthesis of 3-quinolinylbenzotriazole

Under an inert atmosphere, a solution of an equimolar mixture of 2-chloroquinoline **4** (0.244 g, 1.5 mmol) and thiazolobenzotriazole **1** (0.3 g, 1.5 mmol) in pyridine (3 mL) was heated in reflux



Scheme 5. Thermal cyclization of triazolobenzoisoquinoline.

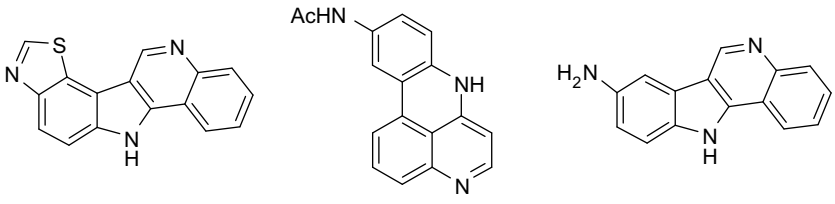


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Fig. 3. Compounds evaluated as potential DNA-intercalating agents.

for 180 min. The mixture was extracted with dichloromethane (15 mL) and washed with water (3 × 10 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by column chromatography (silica gel, dichloromethane) to provide 2 regioisomers **12** and **13** in 15% and 11% yields respectively.

*4.1.4.1. 3-Quinolin-2-yl-3*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**12**).* Yield: 15%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.59 (d, J = 8.8 Hz, 1H, ArH), 8.50 (d, J = 8.8 Hz, 1H, ArH), 8.38 (d, J = 8.0 Hz, 1H, ArH), 8.22 (d, J = 9.2 Hz, 1H, ArH), 8.19 (d, J = 9.2 Hz, 1H, ArH), 7.97 (d, J = 8.0 Hz, 1H, ArH), 7.88 (td, J = 1.2 and 6.8 Hz, 1H, ArH), 7.69 (td, J = 1.2 and 6.8 Hz, 1H, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 153.30, 149.51, 146.77, 144.84, 140.56, 140.07, 136.70, 131.25, 129.85, 128.02, 127.76, 125.23, 119.37, 113.87, 112.76, 109.97, 29.69. IR (KBr, cm^{-1}) 3403, 2238 (CN), 1736, 1723, 1594, 1434, 1263. HRMS (EI) $[M]^+$ ($C_{17}H_8N_6S$): calcd. 328.0512; found 328.0530.

*4.1.4.2. 1-Quinolin-2-yl-1*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole-7-carbonitrile (**13**).* Yield: 11%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.29 (d, J = 9.2 Hz, 1H, ArH), 8.55 (d , J = 8.8 Hz, 1H, ArH), 8.48 (d, J = 8.8 Hz, 1H, ArH), 8.45 (d, J = 9.2 Hz, 1H, ArH), 8.23 (d, J = 8.4 Hz, 1H, ArH), 7.96 (d, J = 8.4 Hz, 1H, ArH), 7.87 (t, J = 7.6 Hz, 1H, ArH), 7.67 (t, J = 7.6 Hz, 1H, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 151.17, 149.83, 146.39, 140.68, 140.54, 139.83, 135.93, 131.04, 128.92, 127.95, 127.52, 127.42, 125.97, 125.46, 116.25, 113.53, 29.70. IR (KBr, cm^{-1}) 3473, 2235 (CN), 1720, 1503, 1437, 1040. HRMS (EI) $[M]^+$ ($C_{17}H_8N_6S$): calcd. 328.0512; found 328.0530.

*4.1.5. General procedure for the preparation of imidazolines (**14**–**17**)*

A stirred mixture of cyanothiazolo derivatives **6**, **8**, **10**, **12** (1 mmol) and ethylenediamine (5 mmol) in anhydrous ethanol (2 mL) under argon was heated under reflux for 2 h. The solvent was removed in vacuo and water (3 mL) was added to the crude residue. The precipitated solid was collected and washed with ethanol (10 mL) to give white imidazoline derivatives **14**–**17**.

*4.1.5.1. 7-(4,5-dihydro-1*H*-imidazol-2-yl)-3-quinolin-4-yl-3*H*-thiazolo[4′,5′;3,4]benzo[1,2-d][1,2,3]triazole (**14**).* Yield: 64%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.14 (d, J = 4.4 Hz, 1H, ArH), 8.88 (d, J = 8.0 Hz, 1H, ArH), 8.29 (d, J = 8.0 Hz, 1H, ArH), 8.14 (d, J = 6.8 Hz, 1H, ArH), 8.12 (d, J = 1.6 Hz, 1H, ArH), 8.08 (d, J = 9.6 Hz, 1H, ArH), 7.88 (td, J = 1.6 and 6.8 Hz, 1H, ArH), 7.76 (td, J = 1.6 and 6.8 Hz, 1H, ArH), 5.76 (s, 1H, NH), 4.1. (t, J = 9.6 Hz, 2H, CH_2), 3.72 (t, J = 9.6 Hz, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 159.47, 153.45, 150.16, 144.42, 143.35, 142.00, 140.47, 140.17, 130.42, 130.07, 128.52, 125.02, 124.31, 120.92, 117.35, 116.15, 100.50, 56.27 ($C_{imidazol}$), 45.35 ($C_{imidazol}$). IR (KBr, cm^{-1}) 3258 (NH), 2867, 1507, 1430, 1288, 987. HRMS (EI) $[M]^+$ ($C_{19}H_{13}N_7S$): calcd. 371.0953; found 371.0974.

*4.1.5.2. 7-(4,5-Dihydro-1*H*-imidazol-2-yl)-3-(2-methyl-quinolin-4-yl)-3*H*-thiazolo[5′,4′;3,4]-benzo[1,2-*d*][1,2,3]triazole (**15**).* Yield: 61%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.77 (d, J = 8.8 Hz, 1H, 4-H or 5-H), 8.18 (d, J = 8.8 Hz, 1H, 4-H or 5-H), 8.13 (d, J = 9.2 Hz, 1H, ArH), 8.07 (d, J = 9.2 Hz, 1H, ArH), 8.01 (s, 1H, 3′-$H_{quinolin}$), 7.82 (t, J = 7.2 Hz, 1H, ArH), 7.67 (t, J = 7.2 Hz, 1H, ArH), 4.15 (s, 2H, CH_2), 3.73 (s, 2H, CH_2), 2.90 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 159.87, 159.46, 159.21, 153.44, 149.90, 144.30, 143.46, 140.40, 130.38, 129.30, 127.50, 124.94, 124.06, 123.98, 119.38, 117.30, 116.99, 100.57, 56.29 ($C_{imidazol}$), 45.35 ($C_{imidazol}$), 25.47 (CH_3). IR (KBr, cm^{-1}) 3418 (NH), 2867, 1726, 1601, 1258. HRMS (EI) $[M]^+$ ($C_{20}H_{15}N_7S$): calcd. 385.1110; found 385.1096.

*4.1.5.3. 7-(4,5-Dihydro-1*H*-imidazol-2-yl)-3-pyridin-4-yl-3*H*-thiazolo[5′,4′;3,4]benzo[1,2-*d*][1,2,3]triazole (**16**).* Yield: 96%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.85 (dd, J = 1.6 and 4.4 Hz, 2H, 2′-H and 6′-H), 8.31 (dd, J = 1.6 and 4.4 Hz, 2H, 3′-H and 5′-H), 8.07 (d, J = 9.6 Hz, 1H, 4-H or 5-H), 7.97 (d, J = 9.6 Hz, 1H, 4-H or 5-H), 4.16 (t, J = 9.6 Hz, 2H, CH_2), 3.71 (t, J = 9.6 Hz, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 159.95, 159.41, 156.79, 153.57, 151.53, 148.45, 146.00, 144.42, 140.62, 125.27, 123.98, 117.29, 114.11, 56.29 ($C_{imidazol}$), 45.35 ($C_{imidazol}$). IR (KBr, cm^{-1}) 3438, 1768, 1745, 1713, 1590, 1382, 806. HRMS (EI) $[M]^+$ ($C_{15}H_{11}N_7S$): calcd. 321.0797; found 321.0772.

*4.1.5.4. 7-(4,5-Dihydro-1*H*-imidazol-2-yl)-3-quinol-2-yl-3*H*-thiazolo[5′,4′:3,4]benzo[1,2-*d*][1,2,3]triazole (**17**).* Yield: 66%. mp >260 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.97 (s, 1H, NH), 8.60 (t, J = 8.4 Hz,

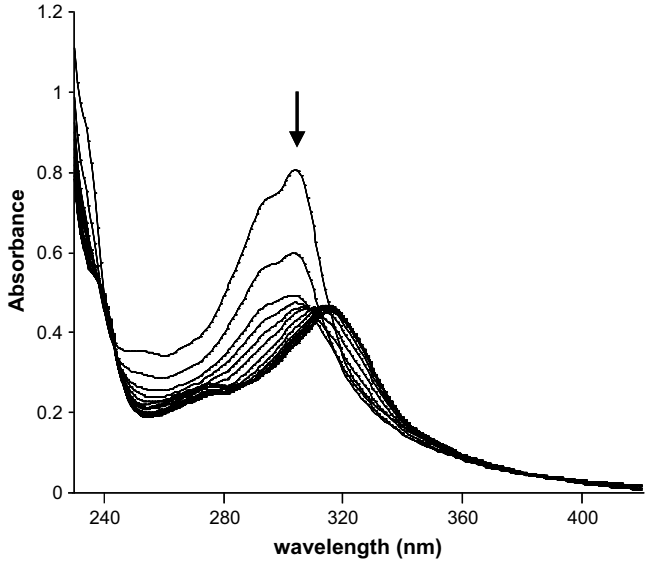

Fig. 4. DNA titration of compound **III** in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). Aliquots of a concentrated calf thymus DNA solution were added to 1 mL of a drug solution (20 μM). The drug/DNA ratio increased from 0 to 20 (top to bottom at 300 nm). Spectra are referenced against DNA solutions of exactly the same DNA concentration and were adjusted to a common baseline.

Table 2
DNA binding and cytotoxicity.

Compound	ΔT_m[a] (°C)		IC_{50}[b] (μM)	
	CT DNA	poly(dAT)$_2$	HL60	HL60/MX2
II	7.2	12.7	17.2	22.6
III	14.5	24.5	4.6	5.5
IV	11.8	20.6	12.5	18.7

[a] Variation of melting temperatures ($\Delta T_m = T_m$ DNA/molecule complex $- T_m$ DNA) at a drug/nucleotide ratio of 1. T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) at 260 nm with a heating rate of 1 °C/min.
[b] Drug concentration that inhibits cell growth by 50% after incubation for 72 h.

1H, ArH), 8.48 (d, $J = 8.8$ Hz, 1H, ArH), 8.38 (d, $J = 8.8$ Hz, 1H, ArH), 8.13 (d, $J = 9.6$ Hz, 1H, ArH), 8.09 (d, $J = 2.0$ Hz, 1H, ArH), 7.96 (d, $J = 8.4$ Hz, 1H, ArH), 7.86 (td, $J = 2.0$ and 7.2 Hz, 1H, ArH), 7.66 (td, $J = 2.0$ and 7.2 Hz, 1H, ArH), 4.16 (t, $J = 9.6$ Hz, 2H, CH_2), 3.72 (t, $J = 9.6$ Hz, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 158.88, 153.38, 149.83, 146.80, 144.50, 139.87, 131.05, 129.83, 128.25, 127.73, 125.39, 125.16, 124.67, 117.99, 113.96, 63.23, 29.60, 14.18, 10.44. IR (KBr, cm^{-1}) 3521 (NH), 2929, 1717, 1595, 1503, 1290, 971. HRMS (EI) $[M]^+$ ($C_{19}H_{13}N_7S$): calcd. 371.0953; found 371.0938.

*4.1.5.5. 9,10-Dimethyl-7*H*-5,7-diaza-benzo[*de*]anthracene (**19**).* Yield: <5% (0.004 g). m.p. 140 °C. ^1H NMR (CD_3COCD_3, 400 MHz) $\delta = 9.25$ (S, 1H), 8. 44 (d, $J = 5.2$ Hz, 1H, 8-$H_{quinolin.}$), 8.07 (d, $J = 7.8$ Hz, 1H, 3-H), 7.91 (d, $J = 8.3$ Hz, 1H, 5-H), 7.77 (d, $J = 5.8$ Hz, 1H, 11-H), 7.73 (dd, $J = 8.3$ and $J = 1.4$ Hz, 1H, 2-H), 7.65 (td, 1H, $J = 8.3$, $J = 7.8$ and $J = 1.4$ Hz, 6-H), 6.83 (d, 1H, $J = 5.2$ Hz, 6-$H_{quinolin.}$), 7.60 (s, 1H, 2-H), 2.33 (s, 6H, CH_3). HRMS (EI) $[M]^+$ ($C_{17}H_{14}N_2$): calcd. 246.11570; found 246.1156.

4.2. Biological activity – materials and methods

4.2.1. DNA and drug solutions

Calf thymus DNA (CT-DNA, Pharmacia) was deproteinized with sodium dodecyl sulfate (SDS, protein content less than 0.2%) and extensively dialyzed against the required experimental buffer. An extinction coefficient of 6600 M/cm was used to measure the nucleotide concentration of DNA solutions. All synthesized compounds, as well as camptothecin and etoposide (Sigma), were dissolved as 10 mM solutions in DMSO. Further dilutions were made in the appropriate aqueous buffer.

4.2.2. Absorption spectrometry and melting temperature studies

Absorption spectra and melting curves were obtained using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. Typically, 20 μM of the various drugs were prepared in 1 mL of BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA, pH 7.1) in the presence or absence of 20 μM of CT-DNA and transferred into a quartz cuvette of 10 mm path length. The spectra were recorded from 230 to 500 nm and are referenced against a cuvette containing the same DNA concentration in the same buffer. For the absorption titration, CT DNA was added gradually from 1 to 20 μM with a spectrum recorded after each addition. To perform the melting temperature measurement, CT DNA (20 μM) was incubated alone (control T_m) or with increasing concentrations of the tested compound in 1 mL of BPE buffer, thus resulting in a drug/base pair ratio of 0.05, 0.1, 0.25, 0.5. The sample was transferred into a quartz cell, and the absorbance at 260 nm was measured every min over the range 20–100 °C with an increment of 1 °C per min. The T_m values were obtained from first derived plots.

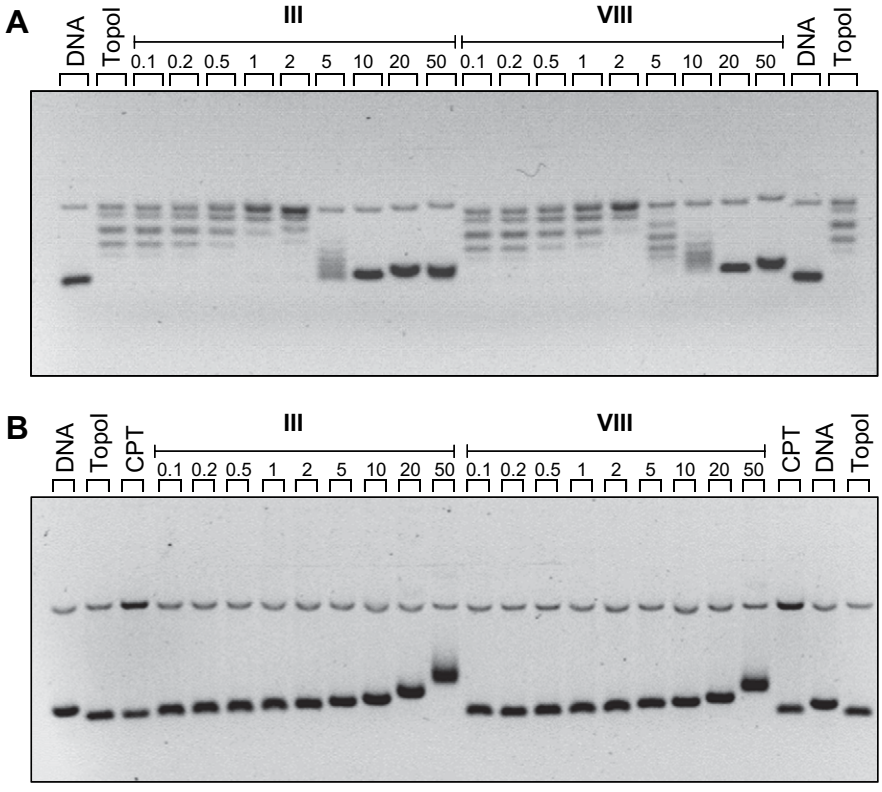

Fig. 5. Topoisomerase I inhibition. Effect of the compounds on the relaxation and cleavage of plasmid DNA by human topoisomerase I. Native supercoiled DNA (0.2 μg) (lane DNA) was incubated with 3 units topoisomerase I in the absence (lane Topo I) or presence of the tested compounds at the indicated concentration (μM). Reactions were stopped with sodium dodecyl sulfate and treatment with proteinase K. DNA samples were separated by electrophoresis. In (A) the gel contained ethidium bromide (1 μg/mL) prior to electrophoresis. In (B) the agarose gel was subsequently stained with ethidium bromide (1 μg/mL) after electrophoresis. Gels were photographed under UV light.

Table 3
Percentage (%) of growth inhibition.

Compound	MDA-MB-231	MCF-7
6	32.4 ± 1.0	34.9 ± 1.8
8	91.9 ± 5.9	87.2 ± 8.3
10	19.8 ± 1.0	30.4 ± 1.2
12	87.4 ± 4.5	95.5 ± 11.5
14	97.3 ± 1.1	95.6 ± 1.0
15	97.4 ± 1.0	98.9 ± 1.1
16	28.5 ± 0.9	41.3 ± 1.8
17	56.4 ± 1.6	51.1 ± 5.3
VIII	85.7 ± 1.7	73.5 ± 2.5

Each compound was tested at 10 μM, after incubation for 72 h.

4.2.3. Topoisomerase inhibition

Supercoiled plasmid DNA (130 ng) was incubated with 4 units of human topoisomerase I or II (TopoGen) at 37 °C for 45 min in 20 μL of relaxation buffer (50 mM tris(hydroxymethyl)aminomethane, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM ATP) in the presence of graded concentrations (from 1.0 to 50 μM) of the tested compound. Reactions were terminated by adding SDS 0.25% and proteinase K 250 μg/mL and incubating at 50 °C for a further 30 min. An amount of 3 μL of the electrophoresis dye mixture was then added to DNA samples, which were then separated by electrophoresis in a 1% agarose gel containing ethidium bromide (1 μg/mL, topoisomerase DNA cleavage gel) or not (inhibition of the relaxation of DNA) at room temperature for 2 h at 120 V. Gels run without ethidium bromide were then stained using a bath containing ethidium bromide. Both gels were finally washed and photographed under UV light.

4.2.4. Cell cultures and anti-proliferative assay

Human HL60 and HL60/MX2 leukemia cells were obtained from the American Tissue Culture Collection. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The cytotoxicity of the tested compounds was assessed using a cell proliferation assay developed by Promega (CellTiter 96 Aqueous one solution cell proliferation assay). Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μL. After 72 h incubation at 37 °C, 20 μL of the tetrazolium dye was added to each well and the samples were incubated for a further 2 h at 37 °C. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm.

MCF-7 and MDA-MB-231 human breast cancer cell lines (LGC Promochem) were grown in a 5% CO_2 humidified atmosphere in DMEM (Gibco) supplemented with 10% heat inactivated FCS and 1% penicillin–streptomycin (Dutscher).

Tested compounds were dissolved in DMSO (Sigma–Aldrich) to obtain 10^{-3} M stock solutions from which further dilutions were made in the cell culture medium. A 50 μL aliquot of medium containing 2×10^{-5} M molecules was added to each well of 96-well plates. After equilibration at 37 °C, 50 μL of a 10^5 cells/mL suspension (5000 cells) were dispensed into all wells of the pre-equilibrated 96-well plate, diluting dithiazoles to a final concentration of 10^{-5} M. After 72 h growth, viable cells were quantified using the MTT cell proliferation assay. Briefly, 20 μL of MTT salt solution (5 g/L in PBS 100 mM pH 7.4, sterile, protected from light) were added to each well, the plates were incubated for a further 4 h to allow MTT metabolism to formazan by the succinate–tetrazolium reductase system active only in viable cells. After incubation, the cell culture medium was removed, and cells were lysed with 100 μL DMSO. Plates were incubated for 10 min at 37 °C to allow solubilization of formazan crystals, and to remove bubbles from the wells. The optical densities were read on a plate reader (VERSAmax, Molecular Devices) at 550 nm. Data were then analyzed to calculate the % of growth inhibition through a comparison of samples with untreated cells (cell culture medium containing 1% DMSO, 0% growth inhibition) and lysed cells (cell culture medium containing 10% SDS, 100% growth inhibition). Data are presented as the mean percentage of growth inhibition ± S.E.M calculated from 24 measures from 3 independent experiments.

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