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Synthesis and antiproliferative activity of some new DNA-targeted alkylating pyrroloquinolines

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Abstract—Two novel DNA-direct alkylating agents, consisting of aniline mustard linked to an angular 3H-pyrrolo[3,2-*f*]quinoline nucleus, were synthetized and assayed for their in vitro antiproliferative activity. Simple convergent synthesis, consisting of separate preparation of 9-chloro-3H-pyrrolo[3,2-*f*]quinoline and *p*-amino-aniline derivatives, and following their linkage by substitution reactions **8a**, **b** and **10**, yielded the corresponding diol derivatives **7b** and **9**. Biological properties were evaluated with respect to cell growth inhibition, ability to form cross-links with DNA, and capacity to give rise to a molecular complex with the macromolecule for **7b**, **8b**, **9** and **10**.

1. Introduction

In the 1990s, DNA-targeted alkylating agents were proposed by researchers in order to avoid some of many drawbacks common to all therapeutically useful DNA-alkylating drugs.[1–4] They were designed as the conjugation of alkyl or aniline mustards to polycyclic planar moieties, chosen among known major DNA-affinity carrier molecules: acridine, antraquinone, quinoline and phenanthridine nuclei. Many series of heterocyclic-linked aniline mustards based on the above nuclei were prepared and evaluated for their in vitro cytotoxicity, in vivo antitumor activity and DNA cross-linking ability: the results and SAR are the subjects of a number of papers.[5–11] Recently, as part of our screening research activity on pyrroloquinoline compounds, we synthesized and studied pyrroloquinoline derivatives as antiproliferative agents, and the tricyclic nucleus showed itself to be a quite good carrier for molecular groups with antitopoisomerase-II activity.[12,13] Continuing our studies with the aim of finding new, less toxic and more selective anticancer drugs, we synthesized and investigated some derivatives characterized by the angular 3H-pyrrolo[3,2-*f*]quinoline chromophore, connected to the aniline bifunctional mustard from known therapeutic alkylating chlorambucil and mephalan.[14] The pyrroloquinoline nucleus may be more advantageous than other known heterocycles because only slightly biologically active by itself, and therefore probably not toxic, very versatile with regard to various easily synthesized geometric structures, and very suitable for various substitutions in order to modulate pharmacodynamic and pharmacokinetic properties of possible active compounds. The antiproliferative activity of **7b**, **8b**, **9** and **10** was evaluated by means of a cell growth inhibition assay on two human tumor cell lines (HeLa, HL-60). The ability to form cross-links with the double helix of DNA was also estimated by thermal denaturation experiments. Lastly, with the aim of evaluating the capacity to form a molecular complex with the macromolecule, linear flow dichroism experiments were performed which, by calculation of the average orientation angle α_L, revealed the binding geometry of the drug–macromolecule complex.

2. Results and discussion

2.1. Chemistry

New bifunctional alkylating agents were obtained by convergent synthesis: intermediates, pyrroloquinoline derivatives and aniline compounds were separately prepared following known multi-step methods, and then allowed to react to furnish final compounds.

Keywords: Pyrroloquinolines; Alkylating agents; Antiproliferative activity; DNA binding.
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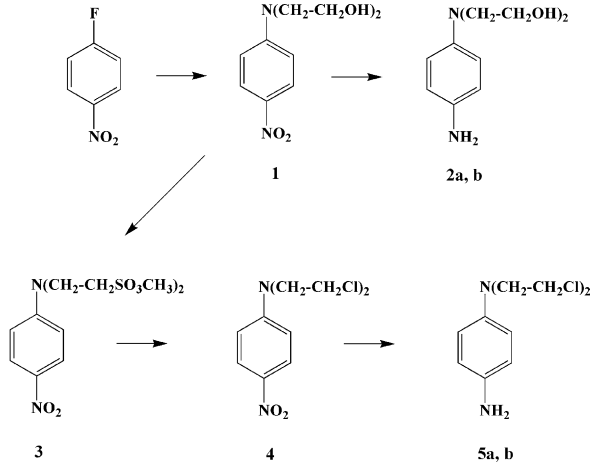
Aniline derivatives **2** and **5** were synthesized (Scheme 1) according to the pathway previously described[15] but starting from 4F-nitrobenzene and diethanolamine in DMSO instead of 4Cl-nitroderivative and diethanolamine; this modification gave a very high yield for **1** and an increase in products **2a**, **b** and **5a**, **b**. Hydrochlorides **2b** and **5b** were prepared in order to restore them, because of their greater stability than that of the corresponding bases. **1** was transformed by reduction with $SnCl_2{\cdot}2H_2O$ whether into the aniline derivative **2a** or into the corresponding mesylate **3** by treatment with MsCl in THF and CH_2Cl_2. The latter was converted to the dichloride-derivative **4** by LiCl in DMF and the aniline **5a** was again obtained by $SnCl_2{\cdot}2H_2O$.

Compound **6** was quantitatively obtained (Scheme 2) from 9-chloropyrroloquinoline[13] by methyl iodide in DMF; compounds **7**–**10** were obtained by means of only one reaction of nucleophilic substitution of the chlorine atom on the pyridine ring of the pyrroloquinoline nucleus with aniline derivative **2a** and **5a**. As shown, either mustard derivatives **8a**, **b** and **10** or the corresponding diols **7b** and **9** were obtained. Hydrochloride **7b** was directly obtained from the reaction; **8b** was prepared in view of biological assays.



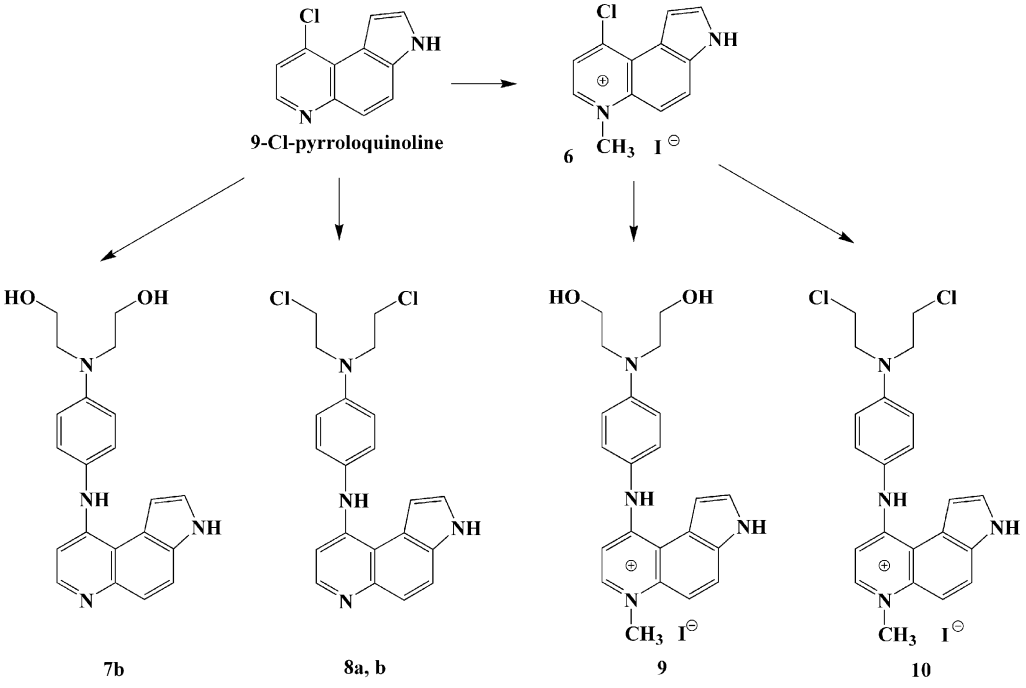
Scheme 1.

2.2. Antiproliferative activity

The antiproliferative activity of **7b**, **8b**, **9** and **10** was tested on human tumor cell lines, HeLa and HL-60, and expressed as IC_{50}, that is the concentration of compound able to cause the death of 50% of cells with respect to the control culture. As reference, we chose the alkylating drug chlorambucil, and **5b** and the pyrroloquinoline because they constitute the two functional moieties belonging to the new synthesized structure. The results (Table 1) clearly indicate that **8b** and **10** are the most active compounds, whereas the two corresponding hydroxyl derivatives, **7b** and **9**, appear to be completely devoid of antiproliferative effect. This behavior reflects the well-known molecular mechanism attributed to the nitrogen mustards, which provides

Table 1. Cell growth inhibition in presence of examined derivatives, **5b**, pyrrolo-quinoline and chlorambucil as reference compounds

Compd	Cell lines IC_{50} (μM)	
	HL-60	HeLa
7b	>20	>20
8b	0.52±0.03	0.66±0.02
9	>20	>20
10	0.45±0.02	0.60±0.06
5b	3.05±0.21	3.35±0.21
Pyrroloquinoline	>20	>20
Chlorambucil	13.2±0.2	20.7±0.2



Scheme 2.

bis(2-chloroethyl)amino functionality for the formation of the aziridinium ion responsible for attack on an external nucleophile.[16–18] In particular, it is interesting to note that the ability of **8b** and **10** to induce a cytotoxic effect appears to be significantly higher with respect to that found for reference compounds. In detail, the IC_{50} values obtained for **5b** in both cell lines are from 5 to 6 times higher than those of the new compounds **8b** and **10**, and for chlorambucil practically 30 times higher. Thus, pyrrolo-quinoline functionality appears to cause a significant increase in the antiproliferative ability of the nitrogen mustard portion, although, as the data of Table 1 show, the pyrrolo-quinoline moiety itself is unable to exert a cytotoxic effect on either of the cell lines examined here.

2.3. Cross-linking

It is well-known that cross-links constitute the main lethal lesion exerted by bifunctional alkylating agents. Consequently, in order to look into the high cytotoxic effect shown by **8b** and **10**, it appeared of interest to evaluate their capacity to give rise to this molecular damage. In detail, the ability to induce cross-links with the double helix of salmon testes DNA was assessed, in comparison with **5b**, by denaturation–renaturation experiments. Figure 1 shows the ability to form cross-links after 10, 30 and 60 min of incubation in the presence of these compounds. In particular, for both **8b** and **10**, the capacity to induce cross-links was significantly higher than that of the reference compound during the overall period of incubation. These results appear to be in agreement with the considerable antiproliferative effect exerted by the new synthesized nitrogen mustard derivatives (see Table 1).

2.4. Linear flow dichroism studies

The remarkable capacity exerted by **8b** and **10** to alkylate DNA, higher than that of **5b**, induced further study of the molecular mechanism explaining their interaction with the macromolecule. In this connection, linear flow dichroism experiments were performed to see how these derivatives give rise to a molecular complex with DNA. Figure 2(a) shows the linear flow dichroism spectra of **10**, at various [drug]/[DNA] ratios, as a representative example. As can be seen, besides the strong negative dichroic signal at 260 nm, attributable to the macromolecule, a significant signal also appears at higher wavelengths (310–470 nm) where only the chromophore belonging to the new derivative can absorb. Similar behavior was also noted for **8b** (spectra not shown). The appearance of this latter signal is believed to indicate the occurrence of a molecular complex with the macromolecule, which allows the small molecule of the drug to become oriented in the flow field. Detectable dichroic signals at wavelengths higher than 260 nm were also revealed for the non-cytotoxic hydroxyl derivatives **7b** and **9** [see Fig. 2(b), showing the spectra of **7b** as an example], whereas no signal appeared for either **5b** or pyrrolo-quinoline (spectra not shown).

Linear flow dichroism spectra also established the geometry by which the interaction takes place. In detail, initial information is obtained from the negative sign of the signal attributable to the drug chromophore (310–470 nm), probably indicating that complexation with the macromolecule occurs through intercalative binding geometry. Nevertheless, confirmation of this

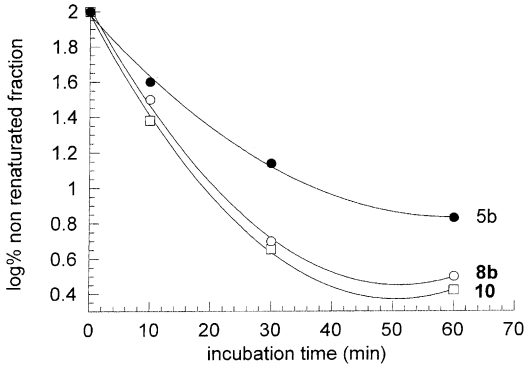


Figure 1. Log% non renaturated fraction of double-stranded salmon testes DNA in the presence of compounds **8b**, **10** and **5b** as a function of incubation time.

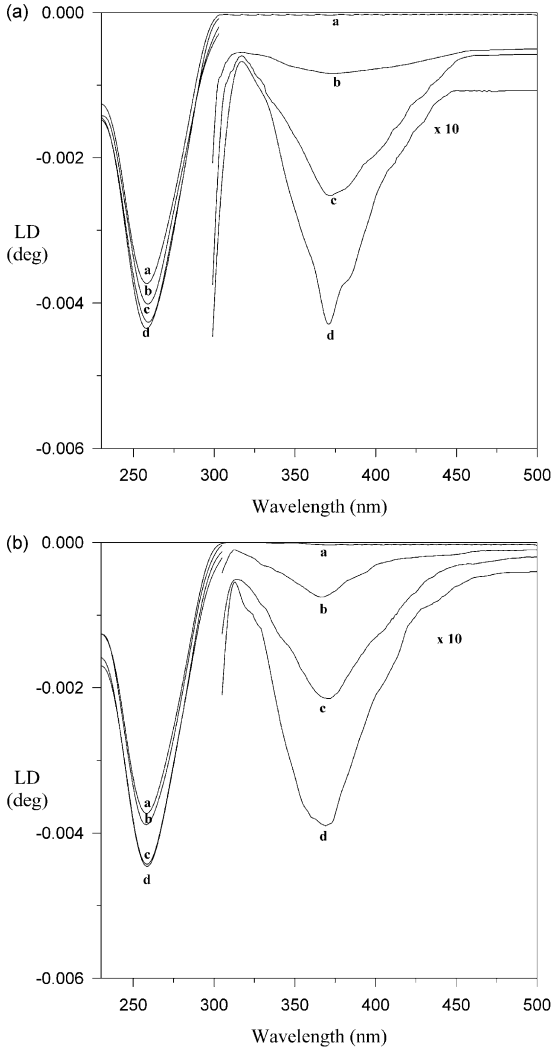


Figure 2. Linear flow dichroism spectra for compounds **10** (a) and **7b** (b) at different [drug]/[DNA]: $a = 0$; $b = 0.02$; $c = 0.04$; $d = 0.08$.

assumption comes from calculation of average orientation angles α_L. Values ranging from 75° to 82° were obtained for **7b**, **8b**, **9** and **10** which are consistent with an intercalative mode of binding,[19] and this means that the planar moiety of the new derivatives assumes perpendicular geometry with respect to the axis of the double helix of DNA.

3. Conclusions

In conclusion, new compounds **8b** and **10** appear to be more active than the nitrogen mustard **5b** in inducing antiproliferative activity on human tumor cell lines. These results, besides the absence of cytotoxic effects of the pyrrolo-quinoline nucleus, make the new chemical structure an interesting tool for the development of DNA-targeted mustards.

4. Experimental

4.1. Chemistry

Melting points were determined on a Gallenkamp MFB 595 010M/B capillary melting point apparatus, and are not corrected. Infrared spectra were recorded on a Perkin-Elmer 1760 FTIR spectrometer as potassium bromide pressed disks; values are expressed in cm^{-1}. UV–vis spectra were recorded on a Perkin-Elmer Lambda UV–vis spectrometer. ^1H NMR spectra were recorded on Varian Gemini (200 MHz) and Bruker (300 MHz) spectrometers, using the indicated solvents; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. *J* values are given in Hertz. In the case of multiplets, the chemical shift quoted was measured from the approximate center. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, using a Perkin-Elmer Elemental Analyzer Model 240B; results fell in the range ±0.4% with respect to calculated values. Mass spectra were obtained with a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and Applied Biosystems Mariner System 5220 LC/Ms (nozzle potential 250.00). Column flash chromatography was carried out on Merck silica gel (250–400 mesh ASTM); reactions were monitored by analytical thin-layer chromatography (TLC) using Merck silica gel 60 F-254 glass plates. Solutions were concentrated in a rotary evaporator under reduced pressure. Starting materials were purchased from Aldrich Chimica and Janssen Chimica (now Acros).

4.1.1. *N*,*N*-Bis(2-hydroxyethyl)-4-nitro-aniline 1. Diethanol amine (2.73 mL, 28.48 mmol) was added to 4-nitrofluorobenzene (1 mL, d = 1.325, 9.38 mmol) in 20 mL DMSO and the solution was heated at 140 °C (oil bath) for 3.5 h. At this time, the starting material had completely disappeared on TLC analysis, and a yellow spot with $R_f = 0.25$ (ethyl acetate) appeared. After cooling at rt, the mixture was evaporated to dryness. Then water was added (50 mL) followed by ethyl acetate. The separated organic phase was washed well with water and worked up to give 1.751 g of pure orange product. Yield 96% (lit.[15] 38%).

4.1.2. *N*,*N*-Bis(2-hydroxyethyl)-4-amino-aniline 2a, b. Nitro-derivative **1** (0.618 g, 2 mmol) was dissolved in 60 mL of concd HCl and $SnCl_2 \cdot 2H_2O$ (2.702 g, 11.97 mmol) was added. The yellow solution was refluxed until the starting material had disappeared (1 h), according to TLC analysis (eluant ethyl acetate, reduced compound with $R_f = 0.2$).

After cooling in an ice bath and diluting with 70 mL H_2O, the resulting mixture was carefully made alkaline to pH 7.5–8 with a 30% NH_4OH water solution. A yellow suspension formed, which was extracted with ethyl acetate, and the workup of the organic phase ended with evaporation to dryness, yielding 0.504 g of the desired aryl-amine **2a**. It quickly darkened in air, whether it was used immediately in the condensation reaction with pyrroloquinolines **7** and **8** or was transformed into its stable di-hydrochloride **2b** by means of the addition of an excess of concd HCl to the concentrated ethyl acetate extract directly. Anal. calcd for $C_{10}H_{16}N_2O_2 \cdot 2HCl$: 44.62 C; 6.74H; 10.41 N; 26.34 Cl; found: 44.32 C; 6.64H; 10.23 N; 26.51 Cl.

The precipitated di-hydrochloride was collected and dried in vacuum. This was again changed into free amine just before starting condensation reactions: it was dissolved in a little water and the solution, made alkaline with 30% NH_4OH, was extracted with ethyl acetate. By the usual quick workup, extract **2a** was again obtained and used.

Yield of **2a** 94%; mp 134–136 °C (lit.[20] 133–135 °C).

4.1.3. *N*,*N*-Bis[2-[(methylsulfonyl)oxy]ethyl]-4-nitro-aniline 3. A solution of diol **1** (0.486 g, 2.15 mmol) in dry THF/CH_2Cl_2 (1:3, 16 mL) was cooled at 0 °C, and Et_3N (0.79 mL, 5.95 mmol) was added followed by methansulfonylchloride (0.56 mL, 7.24 mmol). The mixture was stirred at 0 °C for 10 min, diluted with CH_2Cl_2, and washed many times with cold aqueous $NaHCO_3$ and brine. After drying with anhydrous Na_2SO_4, it was evaporated to give a residue of 0.628 g. Yield 92% (lit.[15] 80%); mp 140 °C (lit.[15] 137–139 °C); $R_f = 0.57$ (TLC, ethyl acetate).

4.1.4. *N*,*N*-Bis(2-chloroethyl)-4-nitro-aniline 4. A solution of dimesylate **3** (0.528 g, 1.66 mmol) in a minimum volume of DMF was added to a LiCl (0.141 g, 3.33 mmol) also in DMF, and the resulting mixture was heated at 110 °C (oil bath) for 5 min. After the solvent had evaporated under reduced pressure, the residue was taken up with water and the resulting suspension extracted with CH_2Cl_2. The organic phase, washed with water and dried with anhydrous Na_2SO_4, was evaporated to give a yellow residue of 0.392 g. Yield 90% (lit.[15] 80%), mp 94–96 °C (lit.[15] 94–95 °C); $R_f = 0.84$ (TLC, ethyl acetate/*n*-hexane).

4.1.5. *N,N*-Bis(2-chloroethyl)-4-amino-aniline 5a, b. To obtain **5a**, **b**, the same procedures as for **2a**, **b** were followed. Starting from nitro-derivative **4** (0.428 g, 1.63 mmol), 0.33 g of mustard **5a** was obtained by reduction with $SnCl_2{\cdot}2H_2O$ (1.86 g, 8.2 mmol). Yield 88%; $R_f = 0.78$ (TLC, ethyl acetate); mp 220 °C dec. (lit.[20] 73–75 °C). This was also immediately used or transformed into its di-hydrochloride **5b**, mp 234–238 °C. Anal. calcd for $C_{10}H_{14}N_2Cl_2{\cdot}2HCl$: 39.24 C; 5.27H; 9.15 N; 46.33 Cl; found: 39.52 C; 5.26H; 9.37 N; 46.12 Cl.

4.1.6. 9-Chloro-6-methyl-3H-pyrrolo[3,2-*f*]quinolinium iodide 6. CH_3I (0.45 mL, 7.23 mmol) was added to 9-chloro-3H-pyrrolo[3,2-*f*]quinoline[13] (0.369 g, 1.82 mmol) dissolved in the minimum volume of DMF and the mixture was heated at 70 °C until the starting material had disappeared, according to TLC analysis (ethyl acetate). During the reaction time (6 h), the brown mixture changed to red. At the end, the solvent was evaporated at 80 °C under reduced pressure. The residue was recrystallized from methanol/ethyl acetate 1:4, yielding 0.408 g of an orange crystalline product. Yield 65%; mp 250 °C; ^{1}H NMR (CD_3OD): δ 4.63 (s, 3H, CH_3), 7.84 (d, 1H, $J_{1,2} = 2.8$ Hz, HC-1), 7.99 (t, 1H, $J_{2,1} = 2.8$ Hz, HC-2), 8.15 (d, 1H, $J_{4,5} = 9.4$ Hz, HC-4), 8.38 (d, 1H, $J_{8,7} = 6.4$ Hz, HC-8), 8.48 (d, 1H, $J_{5,4} = 9.4$ Hz, HC-5), 9.26 (d, 1H, $J_{7,8} = 6.4$ Hz, HC-7), 12.72 (bs, 1H, NH, DMSO); ^{13}C NMR (DMSO-d_6): δ 48.194 (CH_3), 107 (C_1), 112 (C_5 and C_2), 122 (C_8), 123 (C_{9b}), 124.5 (C_{9a}), 125 (C_4), 129 (C_7), 134 (C_{3a}), 144.5 (C_9), 152 (C_{5a}); ms: MH^+(LC–MS) 345.63. Anal. calcd for $C_{12}H_{10}N_2ClI$: 41.83 C; 2.93H; 8.13 N; 10.29 Cl; 36.83 I; found: 41.65 C; 2.98H; 8.08 N; 10.15 Cl; 36.85 I.

4.2. General procedure for synthesis of 9-anilino-pyrroloquinoline derivatives 7 and 8

To a stirred methanol solution of 9-chloro-3H-pyrrolo[3,2-*f*]quinoline[13] (0.251 g, 1.24 mmol), 37% HCl (0.103 mL, 1.24 mmol) was added at rt. After 15 min, a methanol solution of base **2a** or **5a** was added dropwise and the mixture was stirred for 48 h at rt following the ongoing reaction by TLC analysis (ethyl acetate/methanol 7:3 for **7** and ethyl acetate/methanol 9:1 for **8**). At the end, the solvent was evaporated and the residue suitably purified.

4.2.1. 9-[4′-[*N,N*-Bis(2″-hydroxyethyl)-amino]-anilino]-3H-pyrrolo[3,2-*f*]quinoline hydrochloride 7. This compound was obtained pure by simple recrystallization with methyl alcohol. Yield 30%; mp >300 °C; $R_f = 0.15$ (ethyl acetate/methanol 7:4); ms: MH^+(LC–MS) 363.2; UV–vis (ethanol): 212 nm (ε = 41.570), 259 nm (25.930), 292 nm (20.290), 354 nm (12.900); ^{1}H NMR (DMSO-d_6): δ 3.50 (m, 4H, 2×CH_2O), 3.59 (t, 4H, $J = 5.2$ Hz, 2×CH_2N), 4.83 (t, 2H, $J = 5.2$ Hz, 2×OH), 6.70 (d, 1H, $J_{7,8} = 6.9$ Hz, HC-8), 6.87 (d, 2H, $J_{2',3' \text{ and } 5',6'} = 8.9$ Hz, HC-2′ and HC-6′), 7.24 (d, 1H, $J_{2',3' \text{ and } 5',6'} = 8.8$ Hz, HC-3′ and HC-5′), 7.69 (d, 1H, $J_{1,2} = 3.2$ Hz, HC-1), 7.73 (d, 1H, $J_{4,5} = 9.0$ Hz, HC-4), 7.82 (t, 1H, $J = 2.2$ Hz and 2.6 Hz, HC-2), 8.15 (d, 1H, $J_{4,5} = 9.2$ Hz, HC-5), 8.36 (d, 1H, $J_{7,8} = 6.9$ Hz, HC-7), 9.19 (s, 1H, NH), 12.40 (bs, 1H, pyrrolic NH); IR (KBr) 3347, 3235 cm^{-1}. Anal. calcd for $C_{21}H_{22}N_4O_2{\cdot}HCl{\cdot}H_2O$: 60.55 C; 6.05H; 13.46 N; 8.40 Cl; found: 60.61 C; 5.93H; 13.22 N; 8.69 Cl.

4.2.2. 9-[4′-[*N,N*-Bis(2″-chloroethyl)-amino]-anilino]-3H-pyrrolo[3,2-f]quinoline 8. 8a. The residue from the general procedure was dissolved in a little water and the solution, once neutralized with NH_4OH 20%, was extracted with ethyl acetate. Extracts were usually worked up and finally evaporated to yield solid products which were recrystallized with absolute ethyl alcohol. Yield 33%; mp >300 °C; $R_f = 0.39$ (ethyl acetate/methanol 7:3); ms: MH^+ (LC–MS) 399.1; UV–vis (ethanol): 258, 290 and 255 nm; ^{1}H NMR (DMSO-d_6): δ 3.75 (s, 8H, 4×CH_2), 6.75 (d, 1H, $J_{7,8} = 5.6$ Hz, HC-8), 6.82 (d, 2H, $J_{2',3' \text{ and } 5',6'} = 8.8$ Hz, HC-3′ and HC-5′), 7.21 (d, 2H, $J_{2',3' \text{ and } 5',6'} = 8.8$ Hz, HC-2′ and HC-6′), 7.29 (d, 1H, $J_{1,2} = 2.6$ Hz, HC-1), 7.48 (d, 1H, $J_{1,2} = 2.6$ Hz, HC-2), 7.56 (d, 1H, $J_{4,5} = 8.8$ Hz, HC-4), 7.67 (s, 1H, NH), 7.77 (d, 1H, $J_{4,5} = 9.0$ Hz, HC-5), 8.28 (d, 1H, $J_{7,8} = 5.6$ Hz, HC-7), 11.71 (sa, 1H, indolic NH); ^{13}C NMR (DMSO-d_6): δ 42.16 (2×C-1″), 56.36 (2×C-2″), 101.92 (C-8), 104.35 (C-1), 112.41 (C-9), 113.19 (C-9b), 113.65 (C-3′ and C-5′), 119.36 (C-4), 121.04 (C-3a), 127.01 (C-4′), 127.59 (C-2), 127.98 (C-2′ and C-6′), 133.49 (C-1′), 137.51 (C-5a), 140.44 (C-9), 146.17 (C-7); IR (KBr) 3439, 1362 and 732 cm^{-1}. Anal. calcd for $C_{21}H_{20}N_4Cl_2{\cdot}H_2O$: 60.56 C, 5.33H, 13.46 N, 16.81 Cl; found: 60.63 C, 5.01H, 13.27 N, 16.93 Cl.

8b. The corresponding mono-hydrochloride was obtained by addition of an equimolar quantity of HCl to a **8a** ethanol solution. After concentration of the solution, the resulting precipitate was collected and dried. UV–vis (ethanol): 214 nm (ε = 32.090), 258 nm (22.450), 291 nm (15.960), 357 nm (12.600); ^{1}H NMR (DMSO-d_6): δ 3.75 (s, 8H, 4×CH_2), 6.63 (d, 1H, $J_{7,8} = 5.6$ Hz, HC-8), 6.93 (d, 2H, $J_{2',3' \text{ and } 5',6'} = 8.8$ Hz, HC-3′ and HC-5′), 7.32 (d, 2H, $J_{2',3' \text{ and } 5',6'} = 8.8$ Hz, HC-2′ and HC-6′), 7.67 (d, 1H, $J_{1,2} = 2.7$ Hz, HC-1), 7.73 (d, 1H, $J_{4,5} = 9.0$ Hz, HC-5), 7.81 (t, 1H, $J_{1,2} = 2.7$ Hz, HC-2), 8.16 (d, 1H, $J_{4,5} = 8.8$ Hz, HC-4), 8.36 (t, 1H, $J = 6$ Hz, HC-7), 9.21 (s, 1H aminic NH), 12.38 (bs, 1H, pyrrolic NH), 14.32 (d, 1H, $J = 6$ Hz, pyridinium NH). Anal. calcd for: $C_{21}H_{20}N_4Cl_2{\cdot}HCl$: 50.88 C; 4.86H; 13.02 N; 24.81 Cl; found: 50.75 C; 4.93H; 13.02 N; 24.60 Cl.

4.3. General procedure for synthesis of 6-methyl-9-anilino-pyrrolo[3,2-*f*]quinolinium Iodide derivatives 9 and 10

To a solution of 9-chloro-6-methyl-pyrroloquinolinium iodide 6 (0.198 g, 0.57 mmol) in 50 mL DMF, about 0.57 mmol of **2b** or **5b** was added. The resulting solution was heated at 100 °C until the aniline compound had disappeared at TLC analysis (ethyl acetate). After 1 h, the reaction ended, the solvent was removed at 80 °C under reduced pressure, and the residue was recrystallized.

4.3.1. 6-Methyl-9-[4′-[*N,N*-bis(2″-idroxyethyl)-amino]-anilino]3H-pyrrolo[3,2-*f*]quinolinium iodide 9. Yield 94%; mp undetermined. (recrystallized from a DMF/

CH_3OH; 1:3); UV–vis (ethanol): 215 nm (ε = 50.440), 262 nm (28.090), 295 nm (21.475), 364 nm (14.775); ^1H NMR (D_2O): δ 3.66 (t, 4H, J = 5.2 Hz, 2 × CH_2N), 3.79 (t, 4H, J = 5.2 Hz, 2 × CH_2OH), 4.34 (s, 3H, CH_3), 6.98 (d, 1H, $J_{8,7}$ = 7.3 Hz, HC-8), 7.26 (d, 2H, $J_{3',2'\text{ and }5',6'}$ = 8.9 Hz, HC-3′ and 5′), 7.35 (d, 2H, $J_{2',3'\text{ and }6',5'}$ = 8.9 Hz, HC-2′ and 6′), 7.48 (d, 1H, $J_{1,2}$ = 2.5 Hz, HC-1), 7.83 (t, 1H, $J_{2,1\text{ and }2,NH}$ = 1.1 and 2.5 Hz, HC-2), 7.91 (d, 1H, $J_{4,5}$ = 8.6 Hz, HC-4), 8.29 (d, 1H, $J_{5,4}$ = 8.6 Hz, HC-5), 8.42 (d, 1H, $J_{7,8}$ = 7.3 Hz, HC-7); ms: MH^+ (LC–MS) 505.45. Anal. calcd for $C_{22}H_{26}N_4O_2I$: 52.28 C; 5.19H; 11.09 N; 25.11 I; found: 52.40 C; 5.35H; 10.99 N; 25.05 I.

4.3.2. 6-Methyl-9-[4′-[*N*,*N*-bis(2″-chloroethyl)-amino]-anilino]3H-pyrrolo[3,2-*f*]quinolinium Iodide 10. Yield 97%; mp 124 °C (ethanol/diethylic ether); ms: MH^+ (LC–MS) 413.1; UV–vis (ethanol): 216 nm (ε = 54.330), 259 nm (29.530), 294 nm (19.860), 364 nm (18.130); ^1H NMR (CD_3OD): δ 3.82 (td, J = 5.3 and 1.5 Hz, 4H, 2×CH_2Cl), 3.92 (td, J = 1.5 and 5.3 Hz, 4H, 2×CH_2N), 4.32 (s, 3H, CH_3), 6.88 (d, 1H, $J_{7,8}$ = 7.25 Hz, HC-8), 7.00 (d, 2H, $J_{2',3'\text{ and }5',6'}$ = 8.9 Hz, HC-3′, HC- 5′), 7.40 (d, 2H, $J_{2',3'\text{ and }5',6'}$ = 8.9 Hz, HC-2′, HC-6′), 7.60 (d, 1H, $J_{1,2}$ = 3.2 Hz, HC-1), 7.83 (d, 1H, $J_{1,2}$ = 3.2 Hz, HC-2), 7.89 (d, 1H, $J_{4,5}$ = 9.4 Hz, HC-4), 8.29 (d, 1H, $J_{4,5}$ = 9.3 Hz, HC-5), 8.38 (d, 1H, $J_{7,8}$ = 7.35 Hz, HC-7); ^{13}C NMR (CD_3OD): δ 40.59 (CH_3), 113.26 (C-3′ and C-5′), 120.28 (C-3b), 121.02 (C-5,) 127.13 (C-3a), 127.90 (C-2′ and C-6′), 128.12 (C-2), 133.81 (C-4′), 136.91 (C-1′), 143.56 (C-7), 146.65 (C-5), 156.84 (C-9); IR (KBr) 3422, 1363, 726 cm^{-1}. Anal. calcd for $C_{22}H_{24}N_4ICl_2{\cdot}H_2O$: 47.16 C; 4.68H; 10.02 N; 12.65 Cl; 22.65 I; found: 47.15 C; 4.55H; 9.86 N, 12.51 Cl; 22.50 I.

4.4. Inhibition growth assays

HL-60 (human myeloid leukemic cells) were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated fetal calf serum (Seromed); HeLa (human cervix adenocarcinoma cells) were grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (Seromed). 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to both media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

HL-60 cells (3×10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added in complete medium and incubated for a further 72 h. HeLa (3×10^4) cells were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium and various concentrations of the test agents or chlorambucil (Fluka) were added. The cells were then incubated in standard conditions for a further 72 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, that is the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

4.5. Evaluation of interstrand cross-links in vitro

Evaluation of cross-links was carried out by measuring the renaturation capacity of cross-linked double helix after thermal denaturation. Aliquots of aqueous solutions of salmon testes DNA (Sigma Co.), alone and at [nucleic acid]/[drug] = 25, were gently mixed for 30 min; the samples were then thermally denatured (95 °C for 15 min) and quickly cooled in ice. The renaturation capacity of DNA, due to cross-link formation, was investigated by recording absorbance at 260 nm. Data were expressed in terms of non-renaturated fraction of compound–DNA complex relative to native DNA, as suggested by Blais et al.[21] In detail:

$$NRF\% = 100 \times \frac{(A_R - A_N)_{\text{modified DNA}}}{(A_R - A_N)_{\text{native DNA}}}$$

where A_N and A_R are, respectively, the optical densities measured at 260 nm of DNA before heat denaturation and after renaturation. The calculated $NRF\%$ values were expressed as log.

4.6. Linear flow dichroism

Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter, converted for LD and equipped with an IBM PC and a Jasco J interface.

Linear dichroism was defined as:

$$LD_{(\lambda)} = A_{//(\lambda)} - A_{\perp(\lambda)}$$

where $A_{//}$ and $A_{\perp}$ correspond to the absorbances of the sample when polarized light was oriented parallel or perpendicular to the flow direction, respectively. The orientation was produced by a device designed by Wada and Kozawa[22] at a shear gradient of 500–700 rpm.

The reduced linear dichroism was defined as:

$$LD_r = LD_{(\lambda)}/A_{iso(\lambda)}$$

where $A_{iso(\lambda)}$ is the absorbance of the sample in the absence of flow. This quantity may be related to an orientation factor (S) and the angle between the active transition moment in the chromophore and the DNA helix axis, α:[23,24]

$$LD_r = 3/2\left(3\cos^2\alpha - 1\right)S$$

Assuming a value of α = 90° for the DNA base-pair chromophore with respect to a local helix axis, α_L may be evaluated for a given ligand:

$$\alpha_L = \arccos\left[1/3 - (LD_r)_L/3(LD_r)_{DNA}\right]^{1/2}$$

where $(LD_r)_L$ is the reduced linear dichroism of the ligand, $(LD_r)_{DNA}$ is the reduced LD of DNA, and α_L

defines the relative ligand–DNA orientation. For the intercalated system, $(LD_r)_L \approx (LD_r)_{DNA}$ and $\alpha_L \cong 90°$.

Aqueous solutions of salmon testes DNA (1.6×10^{-3} M) in TRIS 10 mM, EDTA 1 mM (pH 7.0) and NaCl 0.01 M were used (ETN buffer). Spectra were recorded at 25 °C at [drug]/[DNA] = 0.02, 0.04, 0.08.

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