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Novel Pyrrolo[3,2-*f*]quinolines: Synthesis and Antiproliferative Activity

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Abstract—Novel pyrrolo[3,2,*f*]quinoline derivatives have been synthesized and tested as antiproliferative agents. They are characterized by an angular aromatic tricyclic system, to which a methyl group can be bound at position 7, and by a methanesulfonanisidide side chain as such, or lacking the *m*-methoxy substituent at position 1. The novel compounds were shown to exhibit cell growth inhibitory properties when tested against the NCI panel of cell lines, in particular those obtained from leukemias. Although the compounds are able to stimulate topoisomerase II poisoning at high concentration, the cell growth inhibition properties do not appear to rest principally on this mechanism of action. Overall, the most active proved to be compound **9,** having the *m*-methoxy substituent typical of amsacrine, followed by the 7-methyl derivative **10** and by the unsubstituted compound **8**. Comparison with previously investigated regioisomers shows modulation of activity dictated by the position and conformational freedom of side-chain groups.

Introduction

Although many effective anticancer agents are available at present, they generally exhibit important side-effects including severe toxicity and resistance.[1,2] Therefore, new compounds are still needed to better understand the drugs' mechanism of cytotoxic action so that more selective therapies can be rationally devised. We recently synthesized and investigated new derivatives,[3] characterized by an angular 9-anilino-3*H*-pyrrolo[3,2-*f*]quinoline planar nucleus connected to the methanesulfon-anisidide residue characteristic of the known anticancer drug *m*-AMSA.[4] They were rather interesting, since their profile of antiproliferative activity was substantially different from that of the parent *m*-AMSA. In particular they were remarkably active against cell lines deriving from solid tumors like CNS-, melanoma- and prostate-derived cells. To further investigate the antiproliferative potential of the pyrroloquinoline family, in this paper we describe the synthesis and cell growth inhibitory activity of pyrrolo[3,2-*f*]quinoline derivatives to which the *m*-AMSA side chain is linked through position 1 (Fig. 1). These congeners of the previously investigated compounds will allow us to assess the pharmacological effects connected to side-chain location.

Results and Discussion

Chemistry

The preparation of the desired compounds was accomplished according to Scheme 1. Compounds **1a,b**, prepared from 5-nitroindole as the starting material by two previously described multistep procedures,[5,6] were dechlorinated to pyrrole[3,2-f]quinolines **2a,b** by catalytic hydrogenation and then transformed into 1-formyl-derivatives **3a,b** with $POCl_3$ in DMF by classical Vilsmeier–Haach reactions. In order to obtain 1-anilinomethyl-substituted compounds, two-step reductive alkylation reactions[7] were performed without separating the anilinium-methyl Schiff base intermediates formed by condensation of **3a,b** with *p*-methanesulfonamidoanilines. $NaBH_3CN$ was used as the selective reductive agent. Hence, compounds **4–7** were obtained in good yields, and easily transformed into the water-soluble dihydrochlorides **8–11**. Yields, melting points, recrystallization solvents, IR and ^1H NMR spectral data of derivatives **2–7** are reported in Table 1; yields, analytical data and MS spectra of compounds **8–11** are reported in Table 2.

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Topoisomerase II poisoning

The extent of DNA damage induced by the test drugs in the presence of human topoisomerase II α was evaluated by gel electrophoresis techniques.[8] The results are summarized in Figure 2. Etoposide (VP-16) and *m*-AMSA were used as reference topoisomerase II poisons and produced the expected cleavage of the nucleic acid in a concentration dependent manner. Compounds **10** and **11** failed to show appreciable DNA-cleavage stimulation in the concentration range 1–100 μM. The same is true for compounds **8** and **9** up to 10 μM when the appropriate lanes are compared to that showing background cleavage (topo II). However, at 100 μM, both congeners exhibited a remarkable level of cleavage stimulation, comparable to *m*-AMSA at the same conditions. However, the cleavage intensity patterns exhibited by the new derivatives are considerably different from the patterns observed with *m*-AMSA. This suggests changes in sequence preference exhibited by pyrrolo-quinolines.

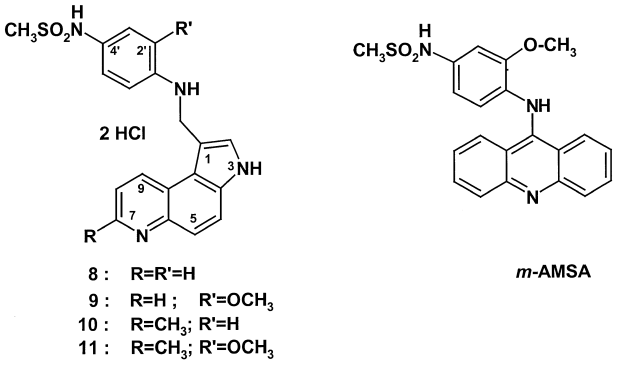


Figure 1. Chemical structure of the test pyrrolo-quinoline derivatives and *m*-AMSA.

From the above data, we can conclude that the topoisomerase-dependent mechanism of DNA damage is appreciably operating only for compounds **8** and **9** at high concentration.

Cell cytotoxicity

Derivatives **8**–**11** were tested as cytotoxic agents in the in vitro primary NCI antitumor screen.[9]

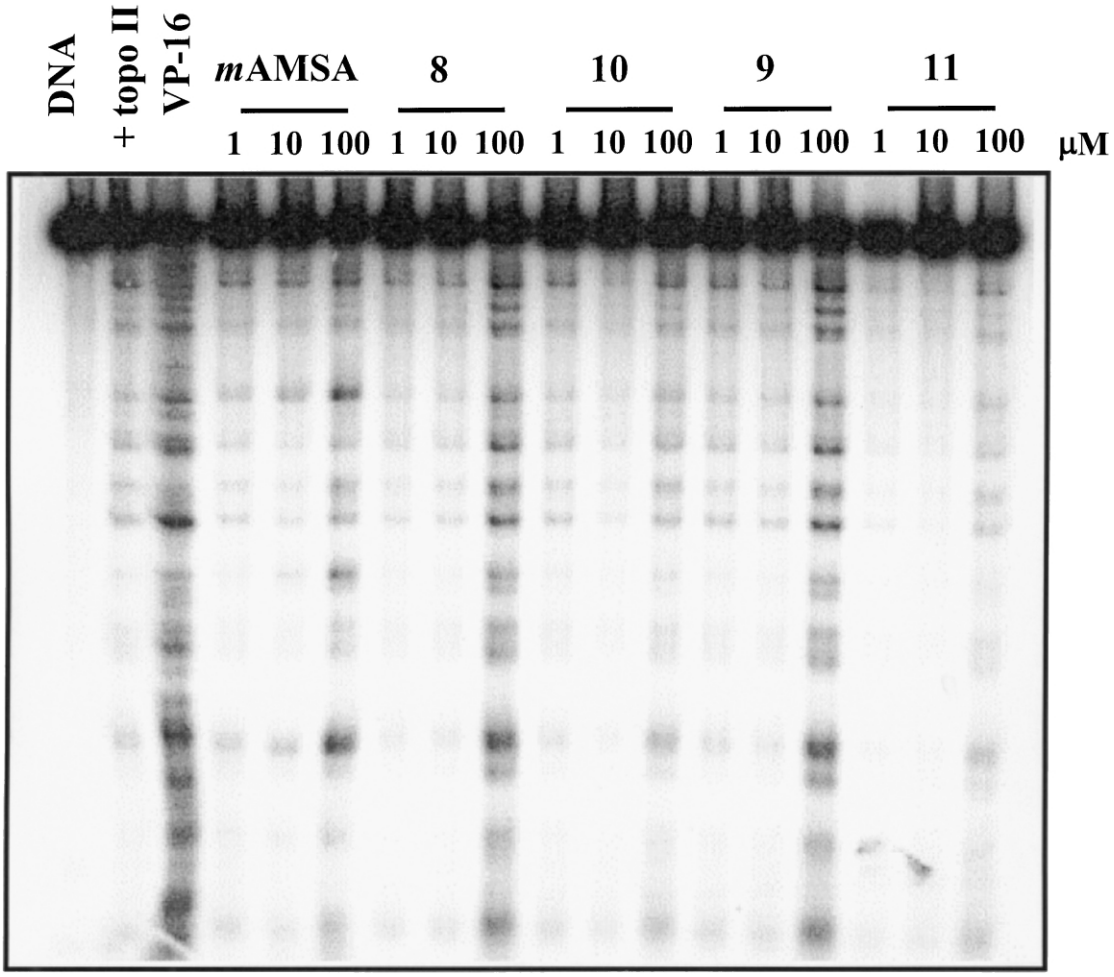
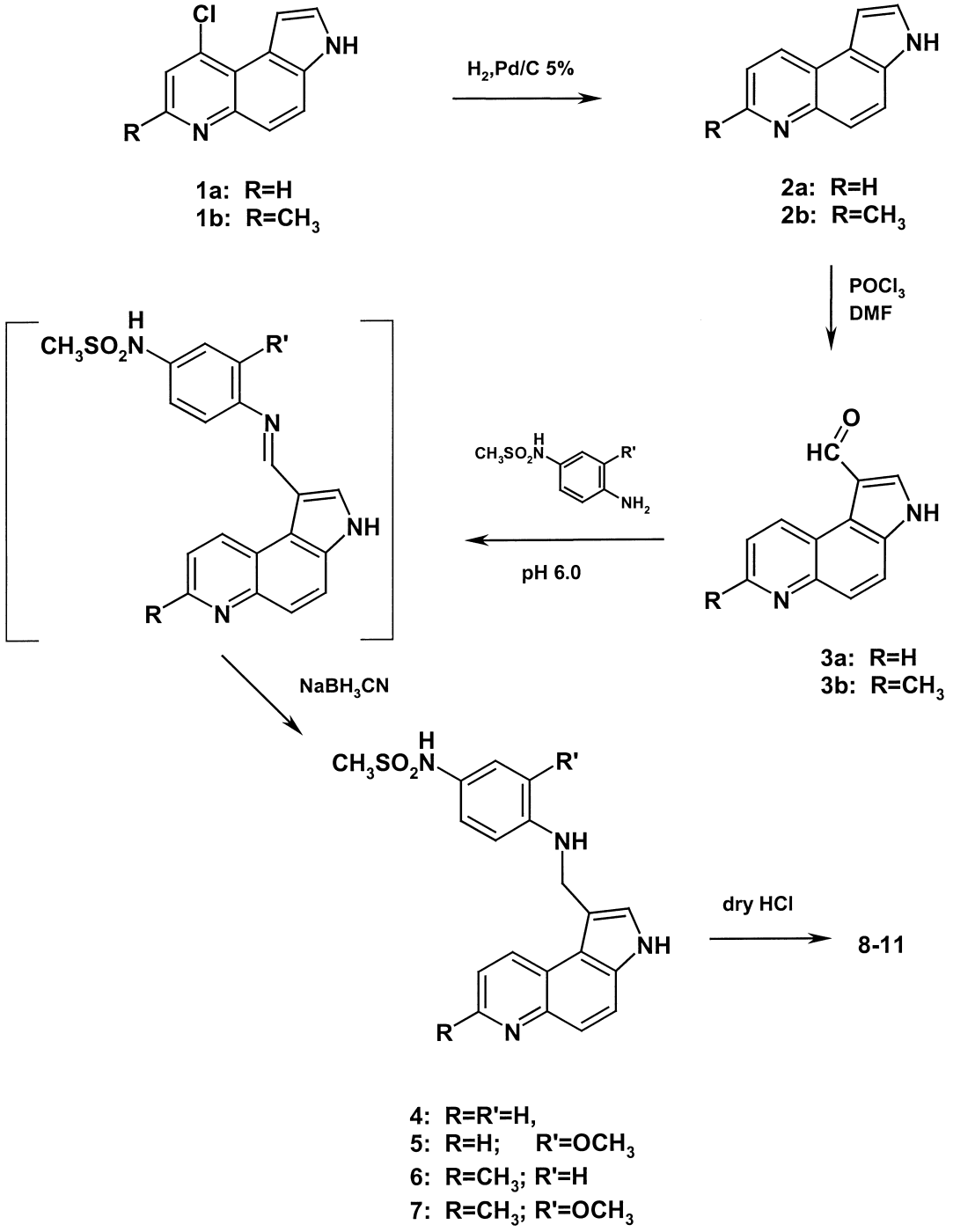


Figure 2. Topoisomerase II-stimulated DNA cleavage. The compounds tested were examined for their ability to stimulate human topoisomerase II-mediated DNA cleavage in the concentration range 1–100 μM. A representative gel showing the activity of the test compounds at three concentrations alongside with the reference compounds *m*-AMSA and etoposide (VP-16, 10 μM) is reported in the figure. The lane labelled DNA contains DNA alone, the lane labeled Topo II contains a mixture of DNA and topoisomerase II at the concentration used in the presence of drugs (1% DMSO in all tubes).

The results are reported in Table 3 as the average of the data obtained with cell lines corresponding to specific types of cancer and as the global average. The measured potency is in the range 0.8–50 μM for the tested compounds. In the case of leukemia cells compound **9** appears to be remarkably effective. In particular, it is found to inhibit tumor cell growth in the nanomolar range using CCRF-CEM or HL-60 ($GI_{50} = 34$ nM) lines. Derivatives **11**, **8**, and **10** are on average 6, 20, and 27 times less potent than **9** against the same leukemia lines.

Potency against solid tumor cell lines is less prominent. Nonetheless, all compounds were active in the micromolar range and did not show particular selectivity for a specific type of tumor (Table 3). The order of effectiveness was in general **9** > **10** > **8** > **11**. In terms of structure–activity relationships, the double 7-methyl-2′-methoxy substitution is clearly decreasing the drug's performance, as well as lack of substitution (see compound **8**). Compounds bearing either the methoxy group or the methyl substituent are more potent, **9** performing somewhat better than **10**.



Scheme 1.

As far as the mechanism of action is concerned, topoisomerase II-mediated DNA damage does not seem to be the major cause of cell death. In fact, considering the potency exhibited by the novel compounds, enzyme-mediated DNA cleavage could hardly be detected in the range of drug concentrations at which remarkable cytotoxic effects are observed. In addition the order of potency does not follow that of cleavage stimulation. In fact, compound **10**, not showing any evidence of topoisomerase-mediated effects, is more effective than compound **8**, the latter behaving as a topoisomerase II poison at high concentration (100 μM). On the other hand, compound **9** is active against leukemia cells at sub-micromolar concentration, while it causes no enzyme poisoning up to 10 μM. Hence, despite the structural similarity with *m*-AMSA shown by the test compounds, they have lost their ability to interfere efficiently with topoisomerase II at cytotoxic concentrations. Moreover, in analogy to our previous findings on the parent 9-substituted derivatives,[3] we tend to rule out a topoisomerase I-related mechanism. As a result, the observed tumor cell growth inhibition by pyrrolo-quinolines should primarily rest on the impairment of other important biological pathways.

A COMPARE[10] analysis was performed using the toxicity data obtained with all new derivatives. This could unravel mechanism(s) of action in common with other previously identified anticancer drugs. The rank of similarity showed that the test drugs can be related to compounds belonging to different chemical classes (see, for example, NSC 71633 and NSC 134120 having a high correlation with compound **9**). In general, they are mono- or bicyclic compounds, with amide, sulfonamide or ester side chains. Amsacrine or related analogues were never found in the list of the 20 most probable hits, which confirms lack of similarity in the mechanism of cytotoxic action.

Finally, it is interesting to compare the newly reported 1-substituted drugs with the previously tested congeners, either substituted at position 9 or having positions 1 and 9 linked through an extra cycle.[3] Indeed, it appears that the position and rigidity of the side-chain modulates drug activity to a significant extent. In fact, the rank of potency in the present series is in favor of 2′

Table 1. Analytical data for compounds **2**–**7**

Compound	Yield %	Mp (°C)	IR (cm^{-1})	^1H NMR δ (ppm)[a]
2a[b]	81	181–182[c]	Ref 12	Ref 12
2b	78	268 (dec)[d]	3357, 3185, 1640, 1373	12.26 (1H, bs, NH), 9.36 (1H, d, $J_{9,8}=8.69$ Hz, HC-9), 8.20 (1H, d, $J_{4,5}=8.94$ Hz, HC-4), 7.98 (1H, d, $J_{5,4}=8.94$ Hz, HC-5), 7.92 (1H, d, $J_{8,9}=8.69$ Hz, HC-8), 7.73 (1H, t, $J_{2,1-2,3}=2.61$ Hz, HC-2), 7.36 (1H, bt, $J_{1,2}=2.42$ Hz, $J_{1,3}=2.16$ Hz HC-1), 2.94 (3H, s, CH_3)
3a	52	280–283[c]	3106, 825, 2734, 1659	9.99 (1H, s, ald.), 9.75 (1H, dd, $J_{9,7}=1.7$ Hz, $J_{9,8}=8.5$ Hz, HC-9), 8.86 (1H, dd, $J_{7,9}=1.7$ Hz, $J_{7,8}=4.2$ Hz, HC-7), 8.54 (1H, s, HC-2), 7.96 (1H, d, $J_{4,5}=9.1$ Hz, HC-4), 7.89 (1H, d, $J_{5,4}=9.1$ Hz, HC-5), 7.61 (1H, dd, $J_{8,7}=4.2$ Hz, $J_{8,9}=8.5$ Hz, HC-8)
3b	62	246–248[c]	3096,2895–2815, 2714, 1669	10.31 (1H, d, $J_{9,8}=8.5$ Hz, HC-9), 10.04 (1H, s, H ald.), 8.70 (1H, d, $J_{2,3}=2.2$ Hz, HC-2), 8.23 (1H, d, $J_{4,5}=9$ Hz, HC-4), 8.13 (1H, d, $J_{5,4}=9$ Hz, HC-5), 7.91 (1H, d, $J_{8,9}=8.5$ Hz, HC-8), 2.89 (3H, s, CH_3)
4	60	184[d]	3475, 3015, 1309	11.60 (1H, bs, NH ind.), 8.99 (1H, s, amide NH), 8.72 (1H, d, $J_{7,8}=4.31$ Hz, HC-7), 8.59 (1H, d, $J_{9,8}=7.9$ Hz, HC-9), 7.84 (1H, d, $J_{4,5}=8.95$ Hz, HC-4), 7.69 (1H, d, $J_{5,4}=8.95$ Hz, HC-5), 7.46 (1H, dd, $J_{8,7}=4.1$ Hz, $J_{8,9}=7.9$ Hz, HC-8), 7.50 (1H, s, HC-2), 7.01 (2H, d, $J_{3',5'}=8.51$ Hz, HC-3′ and HC-5′), 6.73 (2H, d, $J_{2',6'}=8.59$ Hz, HC-2′ and HC-6′), 5.97 (1H, bt, $J_{NH,CH2}=4.76$ Hz, amine NH), 4.57 (2H, d, $J_{CH2,NH}=4.76$ Hz, CH_2), 2.84 (3H, s, CH_3)
5	65	223[d]	3418-3367, 3287, 3126, 2905, 1604, 1147	11.62 (1H, bs, indolic NH), 9.09 (1H, bs, amide NH), 8.73 (1H, d, $J_{7,8}=4.3$ Hz, HC-7), 8.60 (1H, d, $J_{9,8}=7.61$ Hz, HC-9), 7.84 (1H, d, $J_{4,5}=8.96$ Hz, HC-4), 7.7 (1H, d, $J_{5,4}=8.96$ Hz, HC-5), 7.48 (2H, m, HC-2 and HC-8), 6.76 (3H, m, aromatic), 4.77 (1H, t, $J_{NH,CH2}=4.77$ Hz, amine NH), 4.65 (2H, d, $J_{CH2,NH}=4.77$ Hz, CH_2), 3.70 (3H, s,-OCH_3), 2.87 (3H, s, CH_3SO_2)
6	50	220–221[d]	3367-3307, 3066, 1604-1510, 1307	11.53 (1H, bs, indolic NH), 8.99 (1H, bs, amide NH), 8.48 (1H, d, $J_{9,8}=8.46$ Hz, HC-9), 7.78 (1H, d, $J_{4,5}=8.9$ Hz, HC-4), 7.60 (1H, d, $J_{5,4}=8.9$ Hz, HC-5), 7.47 (1H, d, $J_{2,3}=2.28$ Hz, HC-2), 7.35 (1H, d, $J_{8,9}=8.46$ Hz, HC-8), 7.00 (2H, d, $J_{3',5'}=8.71$ Hz, HC-3′ and HC-5′), 6.72 (2H, d, $J_{2',6'}=8.79$ Hz, HC-2′ and HC-6′), 5.95 (1H, t, $J_{NH,CH2}=4.68$ Hz, amine NH), 4.55 (2H, d, $J_{CH2,NH}=4.68$ Hz, CH_2), 2.84 (3H, s, CH_3-SO_2), 2.62 (3H, s, CH_3)
7	52	221–224[d]	3548, 3377, 1313, 1257-1031, 1142	11.53 (1H, bs, indolic NH), 9.09 (1H, bs, amide NH), 8.48 (1H, d, $J_{9,8}=8.6$ Hz, HC-9), 7.78 (1H, d, $J_{4,5}=8.9$ Hz, HC-4), 7.60 (1H, d, $J_{5,4}=8.9$ Hz, HC-5), 7.46 (1H, d, $J_{2,3}=2.48$ Hz, HC-2), 7.35 (1H, d, $J_{8,9}=8.6$ Hz, HC-8), 6.81–6.71 (3H, m, aromatic), 4.72 (1H, bt, $J_{NH,CH2}=4.4$ Hz, amine NH), 4.63 (2H, bd, $J_{CH2,NH}=4.4$ Hz, CH_2), 3.70 (3H, s,–OCH_3), 2.87 (3H, s, SO_2CH_3), 2. 62 (3H, s, CH_3)

[a]Solvent: DMSO-d_6.
[b]Ref 12.
[c]Recrystallized from CH_3OH.
[d]Recrystallized from C_2H_5OH.

Table 2. Analytical data for compounds **8**–**11**

Compound	Yield (%)	$+^{13}$C NMR δ (ppm)[a]	Elemental analysis[b]	Ms (70 eV) *m*/*z* (%)
8[c]	70	39.9 (S–CH_3), 50.0 (CH_2), 110.5 (q, C-1), 115.1 (2CH, C-2′ and C-6′), 121 (C-1′), 122.1 (CH, C-8), 122.6 (2CH, C-3′ and C-5′), 124.7 (CH, C-2), 126.0 (CH, C-5), 126.1 (2q, C-3a and C-3b), 132.57 (CH, C-4), 135.6 (q, C-4′), 137.7 (q, C-9a), 141.1 (CH, C-9), 143.2 (CH, C-7), 145.7 (q, C-5a)	C 49.90 (49.88) H 4.85 (4.52) N 12.25 (12.57) S 7.01 (7.04) Cl 15.50 (15.73)	367 [M^+, 8], 288 (32), 271 (9), 180 (28), 166 (100), 79 (13), 64 (32)
9[c]	53	40.0 (S–CH_3), 50 (CH_2), 57.3 (OCH_3), 105.16 (CH, C-3′), 110.48 (q, C-1), 112.9 (CH, C-5′), 115.15 (CH, C-6′), 121.3 (q, C-1′), 122.11 (CH, C-8), 124.72 (CH, C-2), 126.05 (q, C-3a), 126.12 (q, C-3b), 126.28 (CH, C-5), 132.72 (CH, C-4), 135.91 (q, C-4′), 137.3 (q, C-9a), 141.28 (CH, C-9), 143.17 (CH, C-7), 145.7 (q, C-5a), 154.64 (q, C-2′).	C 49.29 (49.25) H 4.96 (4.68) N 11.50 (11.27) S 6.58 (7.06) Cl 14.55 (14.38)	396 [M^+, 10], 305 (32), 290 (9), 199 (27), 185 (100), 79 (12), 64 (33)
10[c]	45	30.38 (CH_3), 39.78 (S–CH_3), 50 (CH_2), 110.16 (q, C-1), 114.65 (2CH, C-2′ and C-6′), 121.2 (q, C-1′), 122.64 (2CH, C-3′ and C-5′), 123.53 (CH, C-8), 124.54 (CH, C-2), 125.5 (CH, C-5), 125.79 (2q, C-3a, C-3b), 132.70 (CH, C-4), 135.23 (q, C-4′), 137.09 (q, C-9a), 142.25 (CH, C-9), 145.8 (q, C-5a), 160 (q, C-7).	C 50.96 (51.30) H 5.13 (4.89) N 11.89 (11.98) S 6.80 (7.01) Cl 15.04 (14.84)	380 [M^+, 7], 299 (31), 196 (18), 183 (100), 108 (22), 79 (9), 64 (30)
11[d]	40	30.38 (CH_3), 39.98 (S–CH_3), 50 (CH_2), 57.4 (OCH_3), 105.16 (CH, C-3′), 110.1 (q, C-1), 112.93 (CH, C-5′), 115.3 (CH, C-6′), 121.2 (q, C-1′), 123.53 (CH, C-8), 124.54 (CH, C-2), 125.4 (CH, C-5), 126.1 (2q, C-3a and C-3b), 132.72 (CH, C-4), 135.91 (q, C-4′), 137.11 (q, C-9a), 142.28 (CH, C-9), 145.7 (q, C-5a), 154.64 (q, C-2′), 161.2 (q, C-7)..	C 52.18 (51.93) H 5.00 (4.87) N 11.59 (11.40) S 6.63 (6.82) Cl 14.67 (14.45)	411 [M^+, 20], 332 (35), 196 (20), 215 (100), 138 (25), 79 (9), 64 (30)

[a]Solvent CD_3OD.
[b]Calculated values in parentheses.
[c]As the dihydrochloride monohydrate.
[d]As the dihydrochloride.

or 7-monosubstituted drugs, while in the 9-substituted family, lack of substituents at those positions was preferred. Moreover, 2′,7 disubstitution and/or ring closure between position 1 and 9 is confirmed to generate less potent drugs. Hence, the side chain groups need to be more flexible to increase activity. Finally, the preferential and potent action against leukemia cell lines exhibited by compound **9** was not observed before in the pyrrolo-quinoline family. This information, along with the unconventional mechanism of cytotoxicity, renders the novel compound an interesting lead for anticancer drug development.

Table 3. Cell growth inhibition properties of the test pyrrolo-quinolines

Type of cancer cell line[a]	GI_{50} (μM)[b]			
	8	**9**	**10**	**11**
Leukemia (6)	15.7±4.6	0.80±0.76	21.2±1.7	5.1±4.5
Non-small cell lung (8)	32.8±15	21.8±3.1	23.4±12	53.7±14
Colon (6)	24.0±6.7	20.9±3.3	26.3±12	43.3±11
Central nervous system (5)	30.9±13	24.9±6.9	24.1±12	50.1±18
Melanoma (8)	20.1±5.1	17.7±2.1	18.5±2.3	53.0±18
Ovarian (6)	36.7±13	21.7±11.4	23.9±3.4	51.2±21
Renal (8)	24.5±5.0	16.6±11.0	21.0±12	29.5±9.1
Prostate (2)	22.2±4.1	19.4±1.8	21.8±2.8	37.1±5.2
Breast (8)	27.0±9.9	16.1±7.6	24.1±10	51.2±9.8
Average	26.3	14.4	22.9	37.1

[a]Number of tested lines in brackets.
[b]Drug concentration inhibiting tumor cell growth by 50%.

Experimental

Chemistry

Melting points were determined with a Gallenkamp MFB-595-010M Melting Point Apparatus, and are uncorrected.

^{1}H NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer using the indicated solvents and TMS as internal reference. The ^{1}H NMR signals are reported in parts per million (δ, ppm), and are characterized as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). In the case of multiplets, the quoted chemical shift corresponds to the multiplet centre. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Coupling constants are expressed in Hertz (Hz).

Mass spectra were obtained with a Mass spectrometer Mat 112 Varian Mat Bremen.

Elemental analyses were performed at the microanalysis laboratory, Department of Pharmaceutical Sciences,

University of Padova, Italy, using a Perkin-Elmer elemental analysis apparatus model 240B. The results for compounds **2–7** were within ±0.4% of the theoretical values. Data for compounds **8–11** are given in Table 3.

IR spectra were recorded in cm^{-1} with a Perkin-Elmer 1760FTIR spectrophotometer using potassium bromide pressed disks.

Thin layer chromatography (TLC) was performed with Merck silica gel F254 polystyrene-backed plates. Eluents used in TLC analysis: a, (ethyl acetate/*n*-hexane 7:3 v/v), b, (methanol/*n*-hexane 9:1 v/v). Column flash chromatography was performed with Merck silica gel (250–400 mesh ASTM).

Chemicals were purchased from Fluka or Aldrich. *p*-Methansolfonamido-anilines were synthesized in our laboratory according to previous procedures.[3,11]

General procedure for the synthesis of dechlorinated compounds 2a,b. A solution of 9-chloro-pyrrolo[3,2-*f*]quinolines **1a,b**[3] (2–3 mmol) in methanol was slowly added to a 5% palladium on charcoal/methanol suspension saturated with hydrogen and the mixture was hydrogenated at atmospheric pressure at 40 °C until the starting material disappeared according to a TLC analysis. After 3–6 h, the mixture was filtered and the filtrate evaporated to dryness to give almost pure crude solids.

General procedure for the synthesis of 1-formyl derivatives 3a,b. A 6:1 mixture of anhydrous DMF and $POCl_3$ (0.5 mL) was cooled to 10–15 °C by means of an ice/water bath and then a solution of pyrroloquinoline **2** (3–3.5 mmol) was added dropwise in DMF. After stirring for 3–4 h at 35–40 °C, one further mL of $POCl_3$ was added and the stirring continued until the starting compound completely disappeared in the TLC assay. After cooling, the mixture was diluted with iced water, made alkaline with NaOH 2N, extracted exhaustively with ethyl acetate, the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude yellow solids obtained by this procedure were finally recrystallized.

General procedure for the synthesis of 1-(p-methanesulfonamido-aniline) pyrroloquinolines 4–7. A methanol solution of equimolar amounts of 1-formyl-pyrroloquinoline (1–1.5 mmol) and *p*-methanesulfonamido aniline or *o*-methoxy-*p*-methanesulfonamido-aniline was made acidic with acetic acid (pH 6) and stirred at room temperature for a few hours until the starting products disappeared in the TLC test (ethyl acetate/*n*-hexane 7:3 v/v). Subsequently, an excess of $NaBH_3CN$ (2.2 mmol) dissolved in 3 mL of methanol was added and the mixture was stirred for two days at room temperature. A precipitate formed during the reduction, the yield of which was increased by concentrating the solution to small volume and keeping at 4 °C overnight. The solid was collected, washed with water and dried. Crystalline products were obtained by flash chromatography on silica gel, followed by crystallization with suitable solvent systems (Table 1).

Upon bubbling dry HCl through a solution of compounds **4–7** in absolute ethanol, crystalline precipitates formed. They were collected and recristallyzed from methanol–ethanol mixtures to yield the water-soluble pure dihydrochlorides **8–11** (Table 2).

Topoisomerase II cleavage assay

The plasmid pBR322 was cut at the EcoR1 site, precipitated and labelled by fill-in with the Large (Klenow) Fragment of DNA Polymerase I and [α-^{32}P]dATP. DNA was resuspended and treated with Hind III to obtain uniquely end-labeled pBR322. DNA (60 ng) was finally reacted with the indicated concentrations of drugs and 1.2 units of human topoisomerase II-α (TopoGEN, Inc., Columbus, OH, USA) at 37 °C in 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 120 mM KCl, 0.5 mM DTT, 30 μg/mL BSA and 0.5 mM ATP. Following 30 min of incubation at 37 °C, samples were stopped by incubation with 0.5 mg/mL proteinase K and 1% SDS at 60 °C for 3 h, and then loaded on 1% agarose gel (0.089 M Tris-borate pH 8.3, 2.5 mM EDTA) and run for 16 h at 35 volts. The gel was then dried and autoradiographed. The effects of drugs were examined up to 100 μM concentration.

Antitumor screen

The test derivatives were submitted to an in vitro anticancer testing at NCI.[9] The cell panel consisted of 60 lines against which compounds were tested at five concentrations at tenfold dilutions. A 48 h continuous drug exposure protocol was used, and a sulforhodamine protein assay was employed to estimate cell viability or growth. Plot studies were performed for the detection of patterns of interest.

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