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Short communication

Discovery of a new anilino-3*H*-pyrrolo[3,2-*f*]quinoline derivative as potential anti-cancer agent

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Abstract

The newly synthesized 1-[4-(3*H*-pyrrolo[3,2-*f*]quinolin-9-ylamino)-phenyl]-ethanone hydrochloride showed high antiproliferative activity by mixed mechanisms of action. The compound acts by forming an intercalative complex with DNA and inhibiting DNA topoisomerase II (topo II) and by blocking the cell cycle in G_2/M phase. Probable cell death by apoptosis is also suggested by flow cytometry analysis.

Keywords: Pyrroloquinoline derivative; Antiproliferative activity; DNA interaction; Antimitotic effect

1. Introduction

In the field of chemotherapeutic drugs, the search for new, more active, more selective and less toxic compounds is still very intense, and new promising anti-cancer approaches are being tested [1,2]. Currently, combined anti-cancer therapies or multi-acting drugs are clinically preferred to traditional cytotoxic treatment, with the aim of overcoming resistance and toxicity drawbacks. These events often prevent successful treatment and are responsible for reduced survival times [3,4].

In discovering small anti-cancer molecules, a notable role is played by polyheterocyclic structures, and among these, a growing attention focuses on the synthesis and study of the biological properties of compounds containing various combinations of indole or quinoline moieties. Recently, much interest has arisen for the pyrroloquinoline (PQ) nucleus [5,6].

Over the past few years, we have synthesized novel PQ derivatives with remarkable cytotoxic activity [7,8] and reported an advantageous ring-forming method for the synthesis of substituted angular PQs. In particular, we synthesized and studied new derivatives, characterized by a 3*H*-pyrrolo[3,2-*f*]quinoline planar nucleus connected with the methansulfon-anisidine residue characteristic of the known anti-cancer drug *m*-amsacrine [9,10] or with the aniline bifunctional mustard from known therapeutic alkylating chlorambucil and mephalan (Fig. 1) [11]. Interestingly, these compounds are remarkably active against cell lines from solid tumors like CNS-, melanoma- and prostate-derived cells [9]. Continuing this research line, we designed and synthesized other PQ-based molecules modified at the side aniline ring with various substituents, in order to identify new lead compounds. In preliminary in vitro cytotoxic screening assays, one of these turned out, interestingly, to exert high antiproliferative activity against some human tumor cell lines. This paper reports the synthesis of a 9-anilino-3*H*-pyrrolo[3,2-*f*]quinoline derivative, its antiproliferative activity on human tumor cell lines, and its ability to interact with some intracellular targets.

2. Results and discussion

2.1. Chemistry

As shown in Scheme 1, 9-chloro-pyrrolo[3,2-*f*]quinoline was obtained by the multi-step pathway previously reported [9], starting from 5-aminoindole by means of the conventional reaction with diethyl ethoxymethylene malonate [12], which

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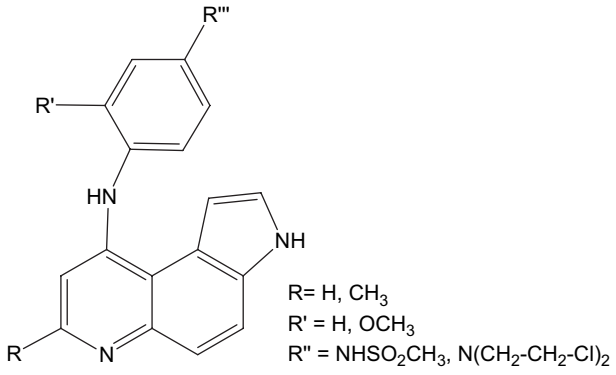
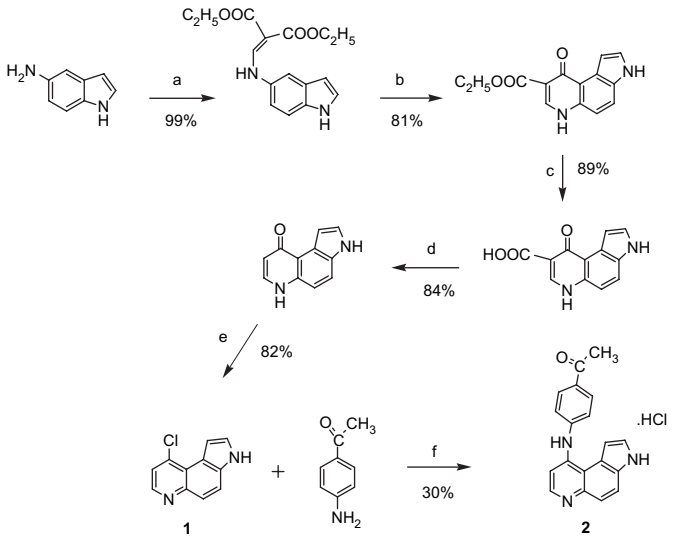


Fig. 1. Some cytotoxic 9-anilino-pyrrolo[3,2-*f*]quinolines previously reported [9,11].

furnished the corresponding enamine derivative. This was thermally cyclized to 8-carbethoxy-pyrrolo[3,2-*f*]quinolin-9-one, which was submitted to alkaline hydrolysis in boiling NaOH 20%, then to decarboxylation in boiling diphenyl ether, and then chlorinated with $POCl_3$ at 70 °C. Nucleophilic substitution of **1** with *p*-amino-acetophenone in the presence of HCl as catalyst afforded 4-acetyl-aniline compound **2**, as monohydrochloride.

2.2. Antiproliferative activity

Evaluation of the biological activity of derivative **2** was carried out by an in vitro antiproliferative assay on four human tumor cell lines: HL-60, HeLa, JR8 and OVCAR-3. HL-60 was derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia [13], HeLa from a biopsy



Scheme 1. Synthetic route for synthesizing anilino-pyrroloquinoline derivative **2**. Reagents and conditions: (a) diethyl ethoxymethylenemalonate, 130 °C, 3 h; (b) diphenyl ether, 250 °C, 15 min; (c) NaOH, 2 N, 3 h, refluxing; (d) diphenyl ether, 250 °C, 25 min; (e) $POCl_3$, 130 °C, 1 h; (f) methanol, HCl cat., 70 °C, 12 h.

of a cervical adenocarcinoma [14], JR8 from lymph node metastasis of poll scalp melanoma [15] and the OVCAR-3 line was established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary [16]. Results, expressed as IC_{50}, i.e., the concentration that is able to cause the death of 50% of cells with respect to the control culture, indicate significant antiproliferative capacity toward all cell lines examined although with different sensitivities (Table 1). In detail, a micromolar IC_{50} value was obtained for HeLa cells; submicromolar and comparable results were found for the others. As the pyrroloquinoline molecule has shown inability to exert antiproliferative action [11], the effect of **2** is attributed to the insertion of the substituted aniline moiety.

2.3. Linear flow dichroism studies

The interesting antiproliferative activity shown by **2** prompted us to study its mechanism of action. A previous study had evidenced the capacity of some derivatives, composed of the PQ chromophore connected to an aniline bifunctional mustard, for remarkable antiproliferative activity, enhanced with respect to reference compounds. In particular, their ability to interact with DNA through an intercalative mode of binding was demonstrated [11]. On the basis of this knowledge, the ability of **2** to give rise to a molecular complex with DNA was assayed by means of linear flow dichroism experiments. The spectra of a solution of salmon testes DNA, alone and in the presence of **2** at a [drug]/[DNA] = 0.08, are reported in Fig. 2. Both the dichroic spectra show a strong negative signal at 260 nm, typical of the macromolecule. Nevertheless, in the presence of PQ derivative **2** (dashed line), a further signal appears at higher wavelengths (300–450 nm), indicating the occurrence of a molecular complex between **2** and DNA. The negative sign also indicates the preferentially parallel orientation of the chromophore to the plane of DNA bases, in agreement with an intercalative mode of binding.

2.4. Inhibition of topo II activity

The ability of derivative **2** to give rise to an intercalative molecular complex with DNA suggested that the synthesized chromophore may interfere with DNA topo II, the enzyme which catalyzes the conversion of pBR322 plasmid DNA from the supercoiled to the fully relaxed conformation [17]. Fig. 3 shows the effect of the new derivative on topo II relaxation ability. In detail, concentration-dependent inhibition was observed: indeed, **2** can partially affect plasmid relaxation at 10 μM, and inhibition is practically complete at 50 μM (lanes c and d, respectively). The high concentration required for

Table 1
Cell growth inhibition in presence of new PQ derivative **2**

Compd.	Cell lines IC_{50} (μM)			
	HL-60	HeLa	JR8	OVCAR-3
2	0.50 ± 0.06	3.4 ± 0.5	0.59 ± 0.07	0.53 ± 0.03

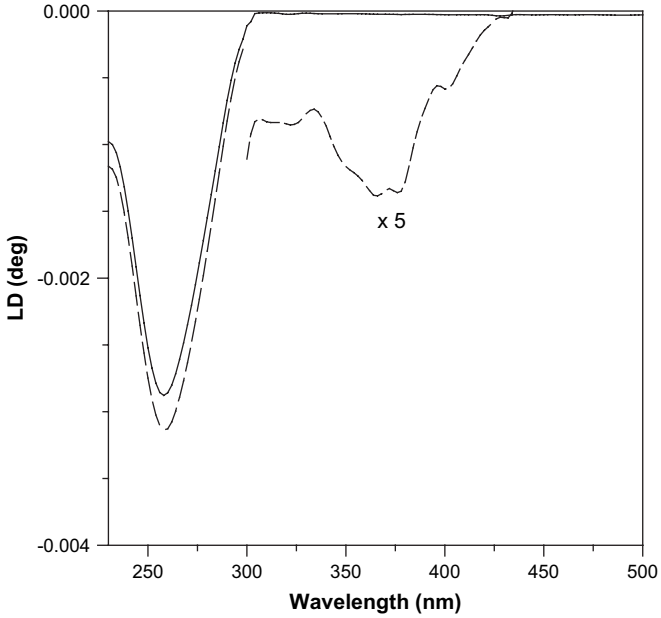


Fig. 2. Linear flow dichroism spectra for salmon testes DNA (straight line) and compound **2** (dashed line) at [drug]/[DNA] = 0.08.

inhibition suggests that this effect is probably not, alone, sufficient to account for the notable antiproliferative activity exerted on human cell lines (see Table 1). This result is in agreement with previous data of some PQ derivatives, whose properties of cell growth inhibition did not appear to be related mainly to inhibition of topo II [9,10]. Further experiments were therefore performed, to identify other possible cellular targets of **2** which could contribute to the overall cytotoxic effect.

2.5. Cell cycle analysis

Flow cytometry analysis was used to examine the effect of the new anilino-PQ **2** on cell cycle progression. The method highlights the effects of drugs on the distribution of cells in specific phases: drugs which interfere with tubulin polymerization block cells in the G_2/M phase, causing an increment of the relative peak in the DNA histogram [18]. Compound **2** was tested on HeLa, JR8, and OVCAR-3 cell lines, at 1−10 μM. It showed a significant dose-dependent antimitotic activity in all the three. For example, in OVCAR-3 (Fig. 4), it caused an increase of the G_2/M peak at 1 μM from 15.3% (Fig. 4a, control) to 31.4% (Fig. 4b), at 5 μM to 57.8% (Fig. 4c) and at 10 μM to 63.7% (Fig. 4d).

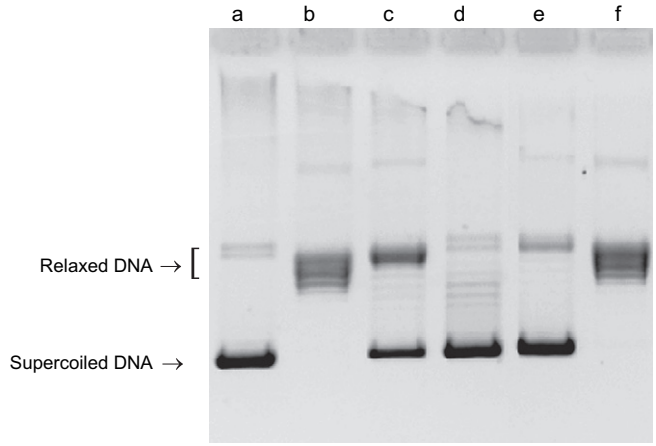


Fig. 3. Effect of derivative **2** on relaxation of supercoiled pBR322 DNA by human recombinant topo II: lanes a, DNA control (no enzyme); lane b, DNA and topo II; lanes c and d, same as lane b with 10 and 50 μM **2**, respectively; lane e and f: same as lane b, with 8 μM *m*-amsacrine and solvent alone, respectively.

Note the good correlation between the cytotoxicity and antimitotic effects of the compound, which was very active in all proliferation assays of all three tested cell lines (Table 1).

Also note the increases in apoptotic peaks at 1 and 5 μM of compound **2** (Fig. 4b and c, 7.6% and 4.3% for cells in sub G_0 state, respectively), in comparison with the control (Fig. 4a, 0.2%). At 10 μM, compound **2** did not show any increase in apoptotic peak (Fig. 4d, 0.5%). Increases at lower doses indicated that apoptosis had occurred, and this specific behaviour − that is, percentage decreases concomitant with dose increases − is in line with the most recent reports of drugs able to target microtubules. At low doses, these drugs act by suppressing spindle-microtubule dynamics and inducing apoptotic cell death; at high concentrations they cause reduction of the microtubule−polymer mass and mitotic arrest in the G_2/M phase [19]. The small observed increases in apoptotic peaks were probably due to the short duration of drug−cell contact (24−48 h).

Flow cytometry results suggest that a possible target for **2** may be microtubules. On the basis of structural considerations, the PQ derivative may interact with the colchicine site on β-tubulin. Indeed, in the molecular structure of **2** pharmacophore elements recurring in tubulin ligands for the colchicine site have been identified, as recently reported by Nguyen et al.[20]: three hydrogen bond acceptors (carbonyl, quinoline nitrogen, amine nitrogen), one hydrogen bond donor (indole nitrogen), hydrophobic centers (aromatic carbons) and one planar group (phenyl). Nevertheless, the exact target site accounting for the antimitotic effect and pro-apoptotic activity of **2** must be confirmed by suitable assays.

3. Conclusions

We have discovered a small PQ molecule which shows cytotoxic activity at submicromolar concentrations in preliminary assays. In the course of a mechanistic study, it turned out to exert antiproliferative activity by multiple mechanisms of action, i.e., intercalation into the DNA double strand, inhibition of topo II, and mitotic arrest in the G_2/M phase. As multi-acting drugs are thought to be suitable for chemotherapy because a single compound can attack tumors through multiple cellular pathways, we believe that 9-(4-acetylanilino)-3*H*-pyrrolo[3,2-*f*]quinoline is interesting enough to prompt further studies of its potential as a lead compound.

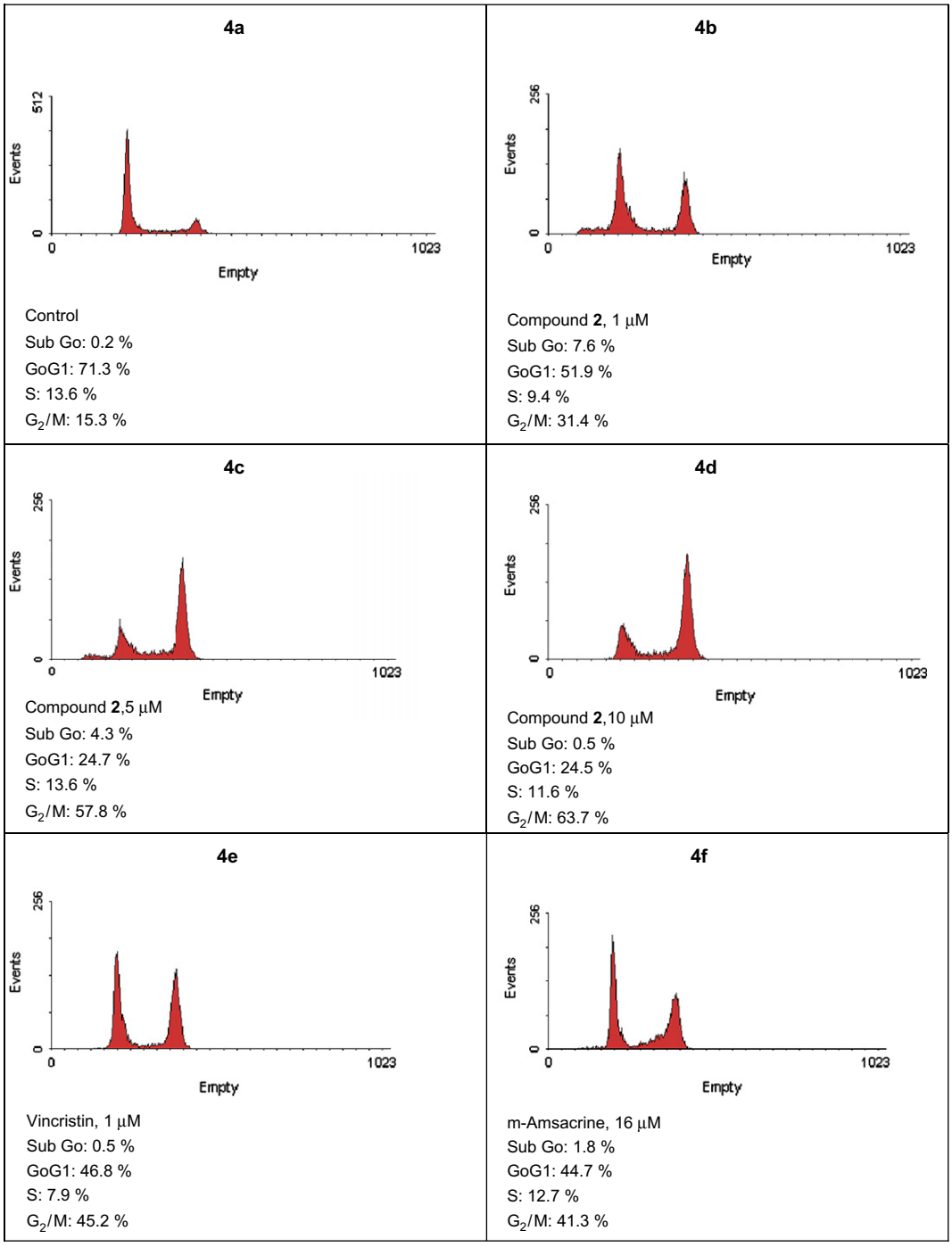


Fig. 4. Cell cycle analysis – effect on cell cycle progression of anilino-PQ derivative **2** at 1, 5 and 10 μM on OVCAR-3 cell line, and vincristine and *m*-amsacrine as references.

4. Materials and methods

4.1. General

Melting points were determined on a Gallenkamp MFB 595 010 M/B capillary melting point apparatus, and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 1760 FTIR spectrometer as potassium bromide pressed discs; values are expressed in cm^{-1}. UV–vis spectra were recorded on a Perkin-Elmer Lambda UV–vis spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300.13 MHz for ^{1}H and 75.04 MHz for ^{13}C, with the indicated solvents. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, on a Perkin-Elmer Elemental Analyzer Model 240B. High-resolution mass spectra

were obtained with an Applied Biosystems Mariner System 5220 LC/Ms (nozzle potential 250.00). Starting materials used in the syntheses shown in the scheme were purchased from Aldrich and Acros Organics.

4.1.1. 1-[4-(3H-Pyrrolo[3,2-f]quinolin-9-ylamino)-phenyl]-ethanone hydrochloride (**2**)

To a solution of 9-chloro-3*H*-pyrrolo[3,2-f]quinoline [5] (**1**) (0.245 g, 1.21 mmol) and HCl 37% (0.1 ml) in 30 ml of methanol, a solution of 4-amino-acetophenone (0.164 g, 1.21 mmol) in 10 ml of methanol was added dropwise under stirring. After about 40 h at 70 °C, the solvents had evaporated and the residue was treated with water. The aqueous solution after extraction with ethyl acetate, which removed unreacted reagents, was concentrated and cooled overnight. The desired product, precipitated as hydrochloride, was collected and recrystallized from hot water. Yield 43%; mp 194 °C; R_f 0.38 (ethyl acetate/methanol 8:2); ^{1}H NMR (DMSO-d_6) δ 2.6 (s, 3H, CH_3), 7.29 (d, 1H, $J_{8,7} = 6.5$ Hz, 8-H), 7.50 (s, 1H, 1-H), 7.68 (d, 2H, $J_{2',3'}$ and $J_{5',6'} = 8.4$ Hz, 2′-H, 6′-H), 7.80 (m, 2H, 4-H and 2-H), 8.12 (d, 2H, $J_{2',3'}$ and $J_{5',6'} = 8.5$ Hz, 3′-H, 5′-H), 8.24 (d, 1H, $J_{5,4} = 8.9$ Hz, 5-H), 8.57 (d, 1H, $J_{7,8} = 6.9$ Hz, 7-H), 9.8 (s, 1H, NH), 12.45 (bs, 1H, pyrrole NH); ^{13}C NMR (DMSO-d_6) δ 26 (CH_3), 103 (9b-C), 104 (9a-C), 113 (1-C), 118 (8-C), 120 (3a-C), 123 (4-C and 2-C), 127 (1′-C), 130 (2′-C and 6′-C), 132 (4′-C), 133 (5-C), 135 (9-C), 138 (5a-C), 143 (3″-C and 5″-C), 154 (7-C), 196 (O-C); HR MS calcd. 337.803; found *m/z* $[M+H]^+$ 302.126; anal. calcd. for $C_{19}H_{16}N_3OCl$: C, 67.56; H, 4.77; Cl, 10.50; N, 12.44; found: C, 67.32; H, 4.79; Cl, 10.45; N, 12.57.

4.2. Cell cultures

HL-60, JR8 and OVCAR-3 cells were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15%, 10% and 10% heat-inactivated fetal calf serum (FCS, Seromed), respectively. HeLa cells were grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated FCS.

Penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin B 0.25 μg/ml (Sigma Chemical Co.) were added to the media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

4.3. Inhibition growth assay

HL-60 cells (4×10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agent were added and incubated for a further 72 h. HeLa, JR8 and OVCAR-3 (4×10^4) 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 agent 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 are expressed as IC_{50} values.

4.4. 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 is oriented parallel or perpendicular to the flow direction, respectively. The orientation was produced by a device designed by Wada and Kozawa [21] at a shear gradient of 500–700 rpm, and each spectrum was accumulated four times and recorded at 25 °C.

A solution of salmon testes DNA (1.6×10^{-3} M) in ETN buffer (containing 10 mM Tris, 10 mM NaCl, and 1 mM EDTA, pH = 7) was used.

4.5. Topo II assay

Topo II activity was measured by the relaxation of supercoiled plasmid DNA. The relaxation assay was performed by incubating in 20 μl reaction volumes of supercoiled pBR322 DNA (Fermentas Life Sciences, 0.25 μg) for 60 min at 37 °C with topo II (USB Corporation, 1 U) in the absence or presence of the test compound. After this, 4 μl of stop buffer and 50 μg/ml of proteinase K (Sigma Chemical Co.) were added and incubated for a further 30 min at 37 °C.

DNA samples were separated by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide 1 μg/ml in TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA, pH = 8), transilluminated by UV light, and fluorescence emission was visualised by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.6. Flow cytometry

HeLa, JR8 and OVCAR-3 cells were cultured for 24–48 h in a drug-free medium or supplemented with test compound (1–10 μM), vincristine sulfate salt (1 μM) or *m*-amsacrine (16 μM). As previously described [22], cells were harvested with a cell scraper, washed twice with PBS, and fixed in 70% cold ethanol (30 min at −20 °C). Cells (10^6) were then washed once in citrate phosphate buffer (0.2 N Na_2HPO_4 and 0.1 M citric acid, 24:1), followed by PBS, and finally incubated in an RNAse solution (100 μg/ml in PBS). After 30 min at 37 °C, the cells were incubated in a propidium iodide solution (PI, Sigma, 100 μg/ml in PBS) at room temperature for a further 30 minutes. To determine the effects of the test compound on cell cycle dynamics, DNA fluorescence was measured by flow cytometry, analyzing at least 15 000 events with Lysis II software. Cells were analyzed using a FACSVantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at 488/525 nm (excitation/emission wavelength).

All experiments were repeated three to four times and DNA content analysis was carried out by means of both logarithmic and linear scales. Results were comparable, irrespective of the scale used, and are shown on a logarithmic scale.

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