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Graphical Abstract

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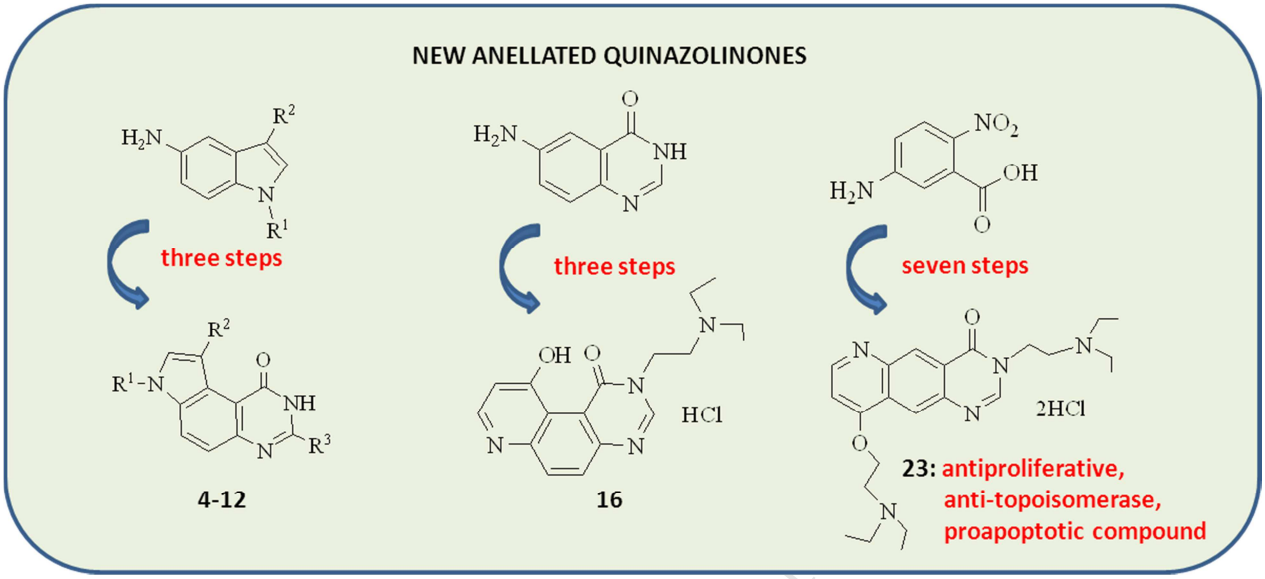

Synthesis of new pyrrolo-, and pyrido-anellated quinazolinones as potential antiproliferative agents

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Abstract

Some pyrrolo[3,2-*f*]quinazolinones (compounds **4**-**12**), two pyrido[3,2-*f*]- and two pyrido[2,3-*g*]quinazolinones (compounds **15**, **16** and **22**, **23**, respectively) were prepared via multistep syntheses. The tricyclic pyrroloquinazolinone nucleus was built starting from 5-aminoindole via a modified reported pathway to form the pyrimidine ring. To synthesize the tricyclic pyridoquinazolinones, new pathways were designed: the angular moiety was obtained starting from 6-aminoquinazolinone to form the pyridine ring and the linear one starting from the 6-aminoquinolinone to form the pyrimidine ring. An interesting antiproliferative activity was found for compound **23**, which was able to form a molecular intercalative complex with DNA and to interfere with the relaxation activity of both topoisomerase I and II. The activation of the apoptotic pathway was also demonstrated.

Keywords: pyrroloquinazolinone; pyridoquinazolinone; antiproliferative activity; DNA intercalation; DNA topoisomerases.

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1. Introduction

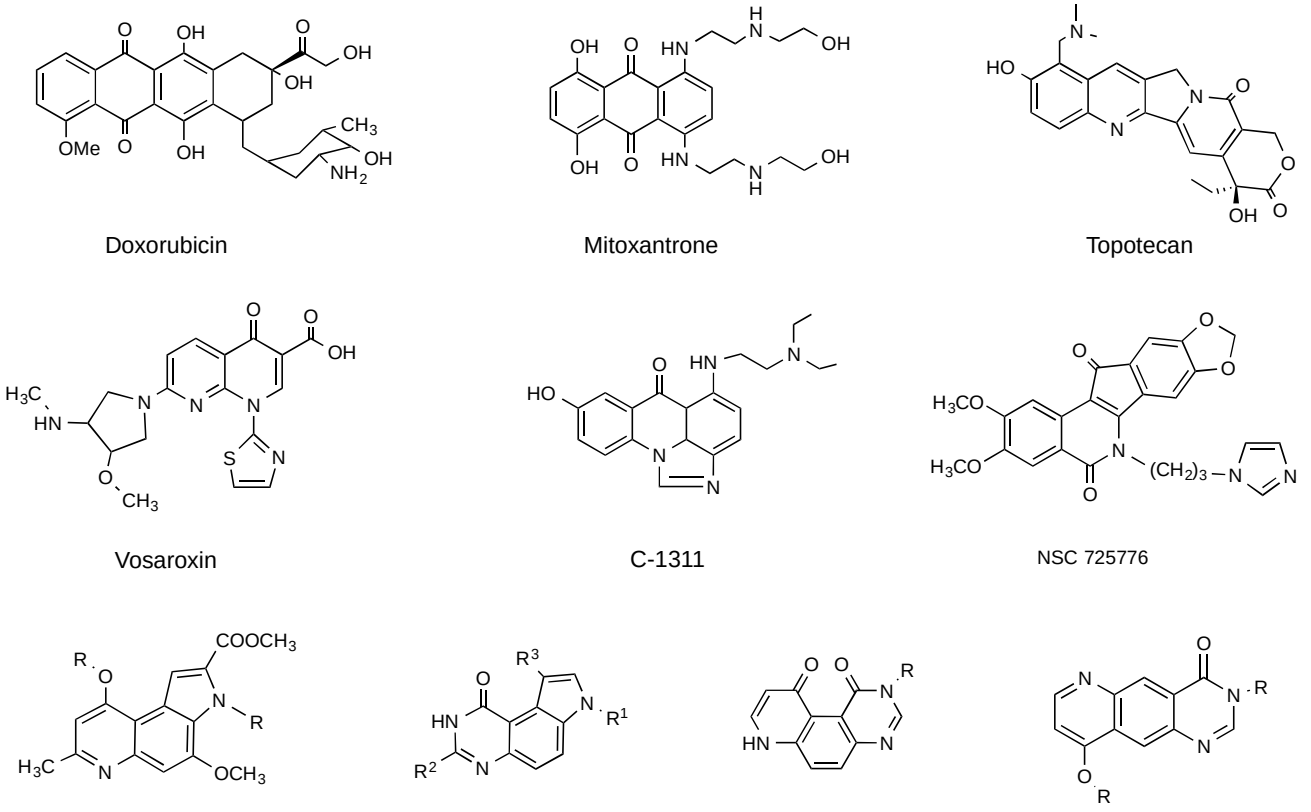
The quinazoline skeleton is found in a number of biologically active molecules and in particular, 4(3*H*)-quinazolinone derivatives display a wide range of biological properties, well documented in the literature.[1] Quinazolinone derivatives have also been developed as antiproliferative agents acting as inhibitors of thymidylate synthase[2] or of tubuline polymerization.[3] Heterocycle-fused quinazolinones are less described and pyrimidoquinazolinones, due to their polycyclic planar structure bearing more or less complex side groups, were proposed as intercalators and topoisomerase II inhibitors.[4,5]

Although many papers describe various polyheterocycle alkylamino-substituted derivatives acting as intercalators and topoisomerase II inhibitors, those focusing on the synthesis and biological profiles of pyrrolo- and pyridoquinazolinones are very few.[6]

Considering the richness of the chemical, physicochemical and pharmacological properties of quinazoline derivatives, the preparation of new fused quinazoline structures remains a worthy area of research.

In the past few years we have focused our interest on the synthesis and biological evaluation of some pyrroloquinolines and pyridocarbazoles as analogues of ellipticine, a well-known intercalating anti-topoisomerase II agent.[7,8] The ability of ellipticine to intercalate into DNA and to alter topoisomerase II activity appears to play an important role in its antineoplastic activity.[9,10]

Recently, we have moved to the synthesis and characterization of some new poly-heterocycles containing the quinazolinone scaffold fused to a pyrrolo or pyridine ring to build both angular and linear tricycles. Figure 1 shows the structures of clinically used prototypic topoisomerase inhibitors (top), inhibitors in clinical development (middle), our previous pyrroloquinoline derivatives,[7] and the new structures proposed in this paper (bottom). The structural similarity among all these compounds is evident: the presence of a planar platform linking side polar groups, two crucial elements for topoisomerase inhibition.[11-14]



R = diethylaminoethyl

R^1 = ethyl

R^2 = dimethylamino, N-methyl-piperazino

R^3 = H, CN, CHO, CH_2OH

Figure 1. Structures of clinically used prototypic topoisomerase inhibitors (top), inhibitors in clinical development (middle), previous pyrroloquinolines,[7] new pyrrolo- and pyrido-quinazolinone structures proposed in this paper (bottom).

2. Results and discussion

2.1. Chemistry

The synthesis of pyrrolo[3,2-*f*]quinazolinone derivatives **4-8** described in Scheme 1 was accomplished adopting a methodology previously reported to form the pyrimidine ring starting from an aromatic amine.[15] Thus, 5-aminoindoles **1-3** obtained by us as previously reported[16] were submitted to a one pot reaction by successive addition of the reagents: ethoxycarbonylisothiocyanate, diethylamine or methylpiperazine, and $HgCl_2$ to the reaction mixture with the indicated stoichiometry. Finally, when the reaction mixture was subjected to thermal cyclization, the 2-alkyl amino-substituted pyrrolo[3,2-*f*]quinazolinones **4-8** were formed by means

of a regio-selective reaction.[17] Indeed, it is worthy to note that the products obtained possess exclusively angular structures, and any product with linear geometry was not observed by proton NMR spectroscopy. The ^{1}H NMR spectra of compounds **4-8** showed two doublets at about δ 7.64 and 7.92 for an *ortho* coupling (J = 8.7 Hz) assigned to H-5 and H-6, respectively, and not two singlets as would be expected for a linear structure. In Scheme 1, the other four pyrroloquinazolinone derivatives described are obtained by modifying compounds **6** and **7** at position 8. Firstly, aldehydic compounds **9** and **10** were prepared by reacting **6** and **7** with $POCl_3$ and DMF, then **9** and **10** were reduced by $NaBH_4$ in methanol to alcoholic derivatives **11** and **12**.

In Scheme 2A and 2B, the total synthesis of two pyridoquinazolinone derivatives, one angular (**16**) and one linear (**23**) is described. The only successful approach starting from **13,** was to react it with the Meldrum's acid in trimethylorthoformate to obtain compound **14** and then thermal cyclization in diphenyl ether (**15**), as described in Scheme 2A. Due to the poor solubility of tricycle **15** in every solvent, the tricycle, without any purification was submitted to alkylation with chloroethyldiethylamine in DMF, and the sole obtained 3-alkylated derivative was treated with HCl gas in absolute ethanol to furnish its mono-hydrocloride **16**.

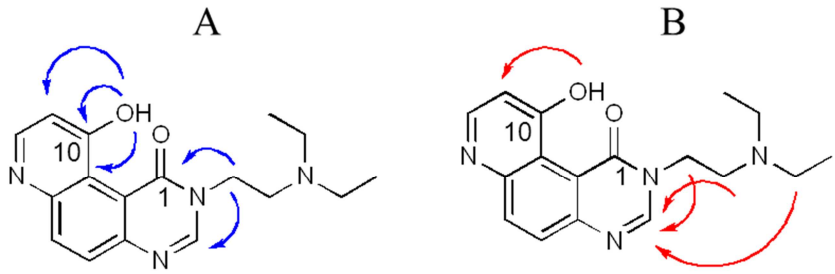


Figure 2. HMBC (A, blue arrows) and NOE (B, red arrows) correlations of compound **16**.

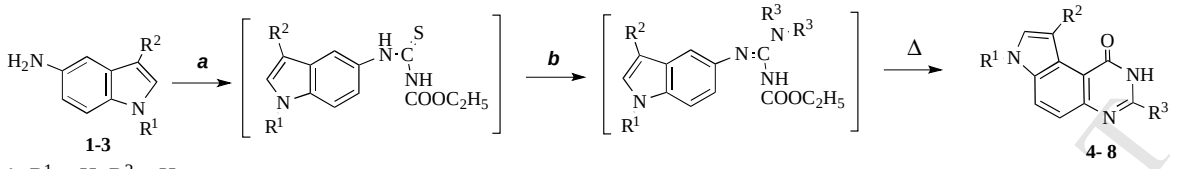
The exact structure of compound **16** was confirmed by HRMS (*m/z* [M+H$^+$] calculated for $C_{17}H_{21}N_4O_2^+$, 313.1759) and 1D and 2D NMR experiments (see the Supplementary Material). The ^{1}H NMR (400 MHz, DMSO-d6) spectrum of compound **16** showed a triplet at 0.81 ppm (6H, J= 7.08 Hz) and a quartet at 2.49 ppm (4H, J= 7.24 Hz) both generated by the ethyldiethylamino chain. Additionally the triplets at 2.75 and 4.34 ppm (J= 5.87 Hz and J= 5.68 Hz, respectively), correspond to the ethylene bridge of the side chain. The presence of two doublets at 7.02 (H-8) and 8.66 (H-9) characterized by a coupling constant of 4.50 Hz is distinctive for the two ortho protons belonging to the pyridine ring. The singlet at 8.67 ppm (H-3) and two doublets at 7.92 (H-5) and 8.30 (H-6) ppm with a coupling constant of 9.10 Hz belong to the quinazolinone nucleus. The sharp singlet at 15.11 ppm is assigned to the hydroxyl proton at position 10 that is de-shielded by the proximity of the carbonyl group. This signal and the presence of two doublets at 7.92 and 8.30 ppm of the ortho coupling H-5 and H-6 are in agreement with a [3,2-*f*] angular geometry of compound **16**.

Both confirmation of specific geometry and complete structural elucidation were realized by means of 2D NMR experiments like NOESY, HSQC and HMBC. By HSQC and HMBC experiments it was possible to determine the site of alkylation, that is the 2-*N* position as well as the HMBC experiments (Figure 2A, blue arrows), that showed a significant correlation between the protons at 4.23 ppm (t, 2H, NC*H*$_2$CH$_2$N), and carbon signals at 149.22 (C-3) and 164.15 (C-1). The hydroxyl proton at 15.11 ppm showed clear correlations with the carbon signals at 111.37 (C-9), 117.77 (C-10a) and 163.39 (C-10) ppm stating an undoubtable presence of a hydroxyl group at position 10.

The NOESY spectrum of **16** (see Supplementary Material) displays correlations between the methylene bridge and the quinazolinone ring system (Figure 2B, red arrows): for example, the proton at 15.11 ppm is next to the proton at 7.02 ppm (H-9) on the same ring, while H-3, H-5 and H-6 showed no NOE effect with other aromatic proton, further evidence of the [3,2-*f*] geometry, an occurrence that cannot be fully understood from the 1D or COSY spectra.

In Scheme 2B, the synthetic route to a linear pyridoquinazolinone starts with the commercial available 5-amino-2-nitrobenzoic acid which was transformed into its methyl ester before reaction with Meldrum's acid and trimethylorthoformate to provide intermediate **18**. This was thermally cyclized at 250 °C (diphenyl ether) to give a mixture of the two isomeric quinolinones **19a** and **19b** in which the desired compound **19a** was present in a higher percentage (63%). Unfortunately, at this stage the two isomers couldn't be separated by any method, thus the mixture was submitted to alkaline hydrolysis (NaOH 20% in methanol) to give the corresponding carboxylic acids. However, only the acid **20** was obtained, while due to its intrinsic chemical stability the isomer **19b** didn't hydrolyze in those conditions, and so was easily removed from the reaction mixture. Successively, the quinolinone **20** was catalytically reduced (H_2, C/Pd 10%) to amino-compound **21** and this was reacted with trimethylorthoformate and CH_3COONH_4 in a MW oven (250W, 5 min)[18] to give the linear pyridoquinazolinone **22**. This was the method giving the best yields of 13%. The linearity of compound **22** was confirmed by ^{1}H NMR spectroscopy, which showed that two singlets at 8.30 and 8.40 δ assigned to H-10 and H-5 respectively were among the other expected signals. Alkylation with chloroethyldiethylamine in anhydrous DMF furnished the bis-substituted derivative that finally was transformed into its dihydrochloride **23** by treatment with HCl dry gas in absolute ethanol. The exact structure of compound **23** was identified by HRMS (*m*/2*z* [M+2H$^+$] calculated for $C_{23}H_{35}N_5O_2^{2+}$, 206.6390; found, 206.6384) that proves the presence of two diethylaminoethyl side chains, and ^{1}H and ^{13}C NMR spectrometry. In the ^{1}H NMR spectrum of **23**, the lack of broad signals of the H-3 and H-6 protons of compound **22** was diagnostic. Additionally, the chemical shifts and the coupling constants of the two doublets due to the H-7 and H-8 (8.03 δ, J=7.44 and 6.07, J= 7.38 Hz) of the pyridone ring in **22** changed into 8.72 δ, J=5.50 Hz and 6.96 δ, J=5.55 Hz of the pyridine ring in compound **23**.

Scheme 1: synthesis of pyrrolo[3,2-*f*]quinazolinone derivatives **4-12**

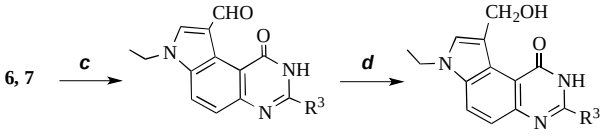


1-3

4- 8

1: R^1 = H; R^2 = H
2: R^1 = C_2H_5; R^2 = H
3: R^1 = C_2H_5; R^2 = CN

4: R^1 = H; R^2 = H; R^3 = $N(CH_3)_2$; 30.0%
5: R^1 = H; R^2 = H; R^3 = $N(CH_2)_2(CH_2)_2NCH_3$; 25.0%
6: R^1 = C_2H_5; R^2 = H; R^3 = $N(CH_3)_2$; 25.0%
7: R^1 = C_2H_5; R^2 = H; R^3 = $N(CH_2)_2(CH_2)_2NCH_3$; 57.0%
8: R^1 = C_2H_5; R^2 = CN; R^3 = $N(CH_3)_2$; 35.0%



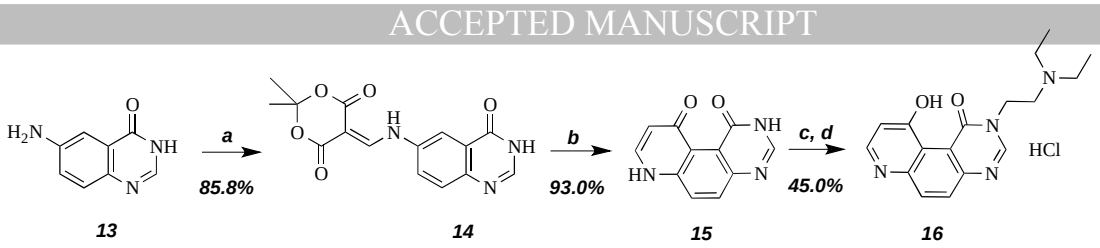
9, 10 **11, 12**

9, 11: R^3 = $N(CH_3)_2$; 87.0%, 52.0%
10, 12: R^3 = $N(CH_2)_2(CH_2)_2NCH_3$; 92.2%, 86.5%

Reagents and conditions:
a) DMF, $SCNCO_2C_2H_5$, r.t., 1 h; b) dimethylamine or methylpiperazine, $HgCl_2$, r.t., 12 h, refluxing 2 h; c) $POCl_3$, DMF, 30-40°C, 4 h; d) methanol, $NaBH_4$, r.t., 3-4 h.

Scheme 2: synthetic routes to pyridoquinazolinone derivatives **16** and **23**.

A

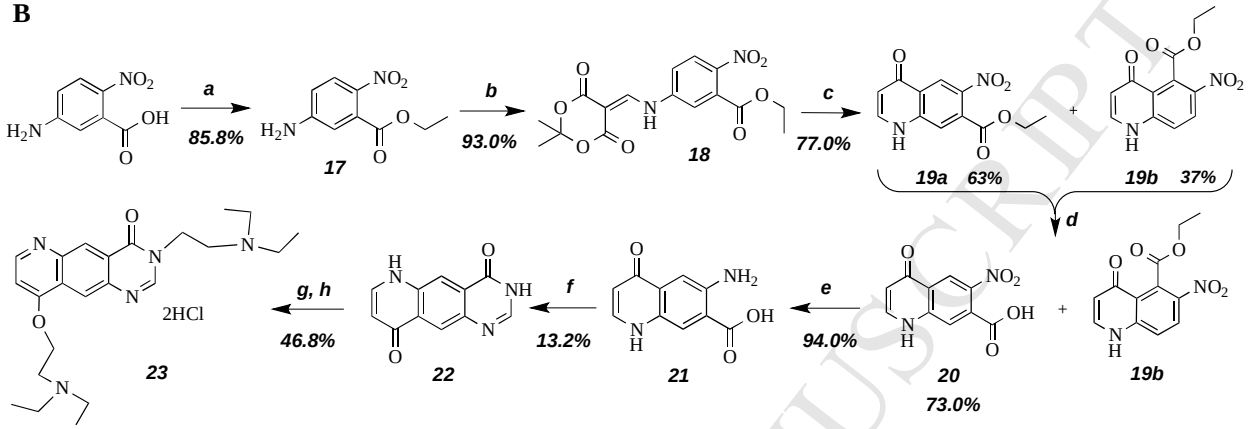


Reagents and conditions:

a) Meldrum's acid, trimethylorthoformate, refluxing, 3+2 h; b) diphenylether, 250° C, 15 min;

c) chloroethylendiethylamine, NaH, anhydrous DMF, TEA, rt, 12 h; d) EtOAc, HCl dry gas;

B



Reagents and conditions:

a) HCl dry gas, EtOH abs, refluxing, 18 h, 89%; b) Meldrum's acid, trimethylorthoformate, refluxing, 2 + 18 h;

c) diphenylether, 250° C, 15 min; d) methanol, NaOH 20%, rt, 24 h; e) C/Pd 10%, H_2, rt, 1 h; f) trimetilorthoformate, CH_3COONH_4, MW, 250W, 5 min; g) choroethylendiethylamine, NaH 60%, anhydrous DMF, r.t., 12 h; h) ethyl acetate, HCl dry gas.

2.2. Biological activity

2.2.1 Antiproliferative effect against human tumor cell lines. The antiproliferative activity of the new pyrrolo- and pyrido-quinazolinone derivatives was evaluated by means of an *in vitro* assay on HeLa (human cervix adenocarcinoma), H460 (large cell lung carcinoma) and MSTO-211H (human biphasic mesothelioma) cell lines. The results were expressed as GI_{50} values, which is concentration (μM) of derivative able to induce 50% of growth inhibition with respect to the control culture. The ellipticine was taken as reference compound.

As regards the pyrroloquinazolinones **4**-**12**, the most active derivatives appear to be **11** and **12**, both characterized by a hydroxymethyl side chain in position 3 of the pyrrole ring. In detail, while **4**-**10** do not affect cell growth up to 50 μM concentration, for **11** and **12** detectable GI_{50} values, ranging from 16.0 to 49.0 μM, are found.

Inside the pyridoquinazolinone series (compounds **15**, **16**, **22** and **23**), the derivative **23** shows a notable antiproliferative activity, with low- (2.9 ± 0.9 μM on HeLa) or sub-micromolar (0.38 ± 0.04 μM and 0.42 ± 0.06 μM on H460 and MSTO-211H, respectively) GI_{50} values. Interestingly, **23** shows cytotoxicity to MSTO-211H cells, higher with respect to that exerted by the reference drug, ellipticine. For this latter drug indeed, a GI_{50} value of 0.77 ± 0.14 μM is obtained. Otherwise, all other synthesised pyrido-quinazolinones do not exert any significant antiproliferative effect, having

GI_{50} higher than 50 μM in all cell lines taken into account. In particular, by comparing the unsubstituted pyridoquinazolinone **22** with **23**, this latter characterised by two diethylaminoalkyl side chains, it could be affirmed that they are essential for the occurrence of an antiproliferative effect, as already demonstrated for some benzothiopyranoindole derivatives.[19]

2.2.2 Determination of apoptosis. To evaluate cell death mechanism, flow cytometry analysis was performed with the most sensitive H460 cells, by using Annexin V-FITC and DNA-specific dye propidium iodide labelling. The obtained results are shown in Figure 3A as dot plots, and in Figure 3B the corresponding percentage are depicted as histograms. By increasing the concentration of **23** from 0 to 40 μM, a decrease in the percentage of viable cells from about 90% to about 75% takes place. This decrease is paralleled by the enhancement of the apoptotic cells, in both early and late apoptosis, that becomes about 20% at the highest concentration. Otherwise, at all considered concentrations, the percentage of cells undergoing a necrosis process is practically negligible and constant (less than 6%).

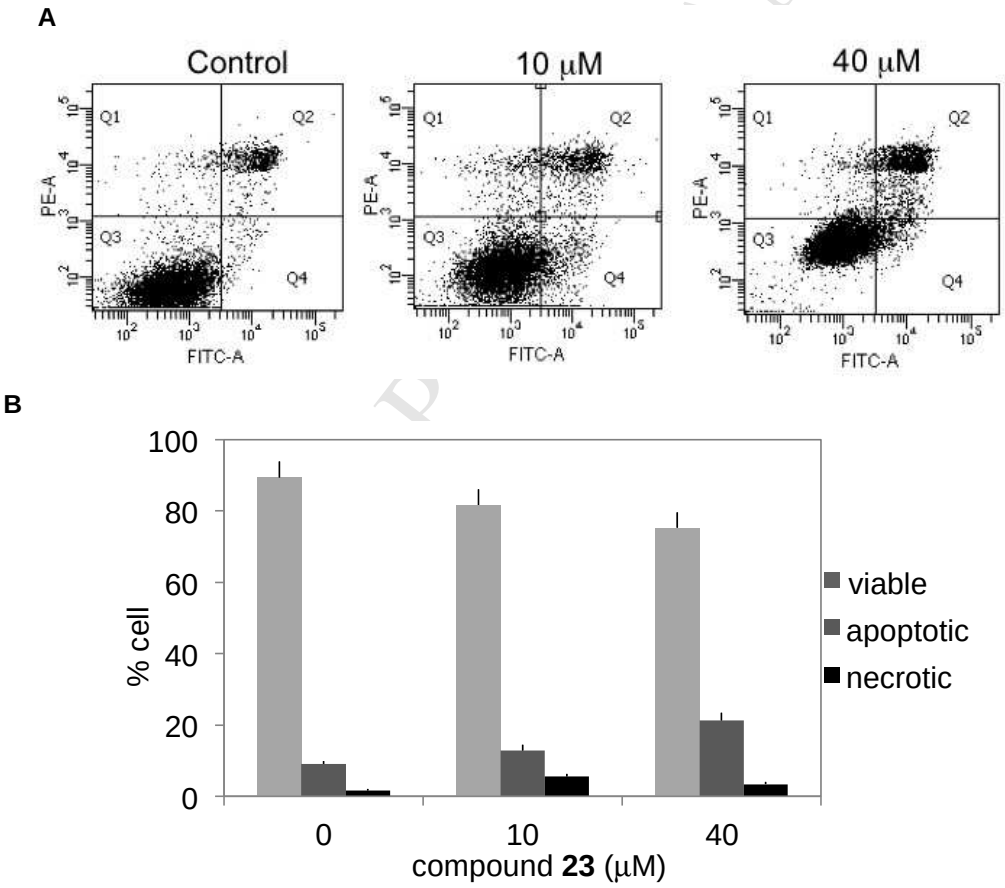


Figure 3. Flow cytometry analysis of cell death induced by **23** (A) Dot plots of the flow cytometry analysis performed on H460 cells labelled with Annexin V-FITC and propidium iodide after 18 h of

treatment with different concentrations of **23**. (B) The histograms show the percentage of viable (Q3), early and late apoptotic (Q4+Q2) and necrotic (Q1) cells. Values are the mean ± SD of four independent experiments.

2.2.3 Interaction with DNA. The presence of the planar pyridoquinazolinone chromophore suggested that the antiproliferative effect exerted by **23** could result from the ability to form an intercalative complex with DNA. Moreover, according to previous results,[19] the presence of diethylaminoalkyl side chains, thanks to the basic nitrogen, protonated at physiological pH, could allow an easier interaction between the nucleic acid and the compound.

To pursue this question, flow linear dichroism (LD) experiments were performed in the presence of salmon testes DNA. Figure 4 shows the UV-vis spectrum of derivative **23** (A) and LD spectra of a DNA solution in the presence of **23** at different [drug]/[DNA] ratios (B).

The LD spectrum of the macromolecule shows the typical negative signal at 260 nm, due to the absorption of nucleotide base pairs, in accordance with previous data.[8] The addition of the test compound induces the occurrence of a dose-dependent negative signal at higher wavelengths (about 320-420 nm) at which only the pyridoquinazolinone chromophore can absorb. Because the small pyridoquinazolinone molecule cannot itself become oriented in the flow field, the presence of a signal at 320-420 nm suggest that **23** is able to form a complex with DNA. The negative sign of this signal indicates an orientation of the chromophore parallel to the plane of DNA base pairs, thus it can be concluded that the complexation occurs through an intercalative mode of binding.

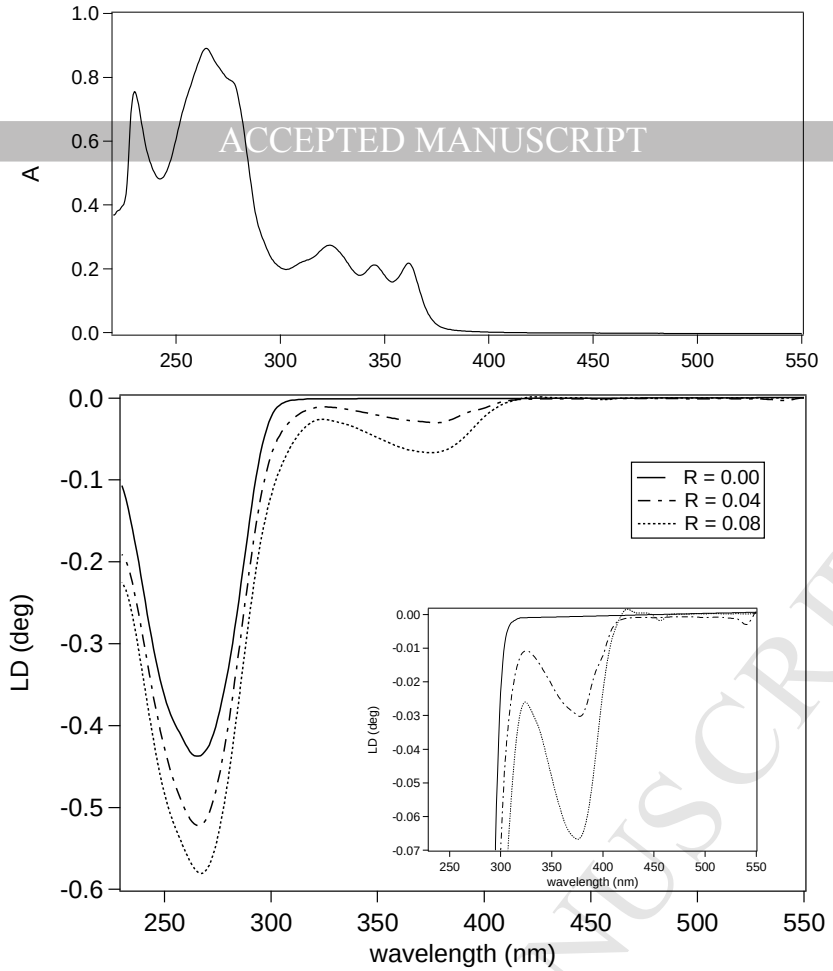


Figure 4. Absorbance (A) and linear dichroism (LD) spectra of compound **23** at different [drug]/[DNA] ratios (R) = 0, 0.04 and 0.08. [DNA]=1.9x10^{-3} M.

2.2.4 Effect on topoisomerases. A number of studies highlighted topoisomerases as the biological targets of many intercalative anticancer agents.[14,20,21] In particular, several clinically used anticancer drugs, designed as topoisomerase poisons, promote the stabilization of the enzyme-mediated DNA cleavage, while some agents, generally referred as catalytic inhibitors, interfere with a step of the catalytic cycle.

In this connection, first of all we evaluated the effect of **23** on the catalytic activity of both topoisomerase I (Fig. 5A) and topoisomerase II (Figure 5B). The incubation of supercoiled DNA (lane DNA) with topoisomerase I (lane topo I) induces the relaxation of supercoiled DNA as demonstrated by the appearance of a series of topoisomers. In the presence of increasing concentrations of the tested pyridoquinazolinone, from 0.5 to 2.5 μM, a concentration-dependent disappearance of the bands corresponding to the relaxed forms occurs, indicating an inhibition of the relaxation activity of the enzyme. A similar behaviour can be observed when supercoiled DNA is incubated in the presence of topoisomerase II (Figure 5B). Indeed, in these experimental conditions, the enzyme promotes the relaxation of supercoiled DNA (lane topo II), and the addition of **23** causes a concentration-dependent decrease of topoisomers. It is noteworthy that in both cases,

by increasing the concentration of **23**, a decrease in the intensity of DNA bands can be observed, and, moreover, at very high concentrations (10-20 μM) a decrease in electrophoretic mobility of the band corresponding to supercoiled DNA occurs. These results suggest that the inhibition of topoisomerase I and II relaxation activity could be due to DNA intercalation rather than through a poisoning effect.

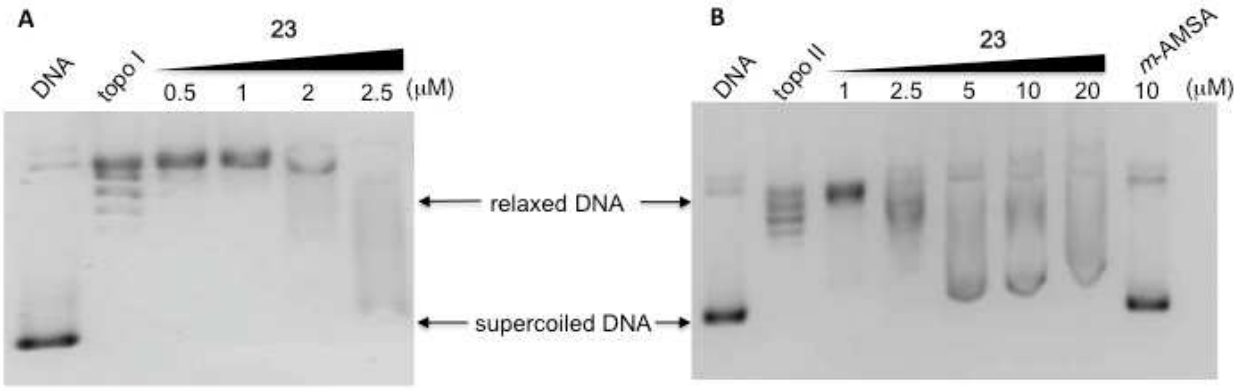


Figure 5. Effect of **23** on relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase I (A) and II (B).

Actually, derivative **23** does not promote DNA cleavage by topoisomerase I or topoisomerase II in contrast to camptothecin and *m*-AMSA, respectively, taken as reference (Figure 6A and B). Indeed, the intensity of the band corresponding to nicked DNA in the presence of topoisomerase I (Figure 6A) and linear DNA for topoisomerase II, (Figure 6B) is not amplified by the addition of **23**. Otherwise, pyridoquinazolinone derivative is able to induce a marked decrease in the electrophoretic mobility of relaxed DNA that can be related to an effective intercalative ability.

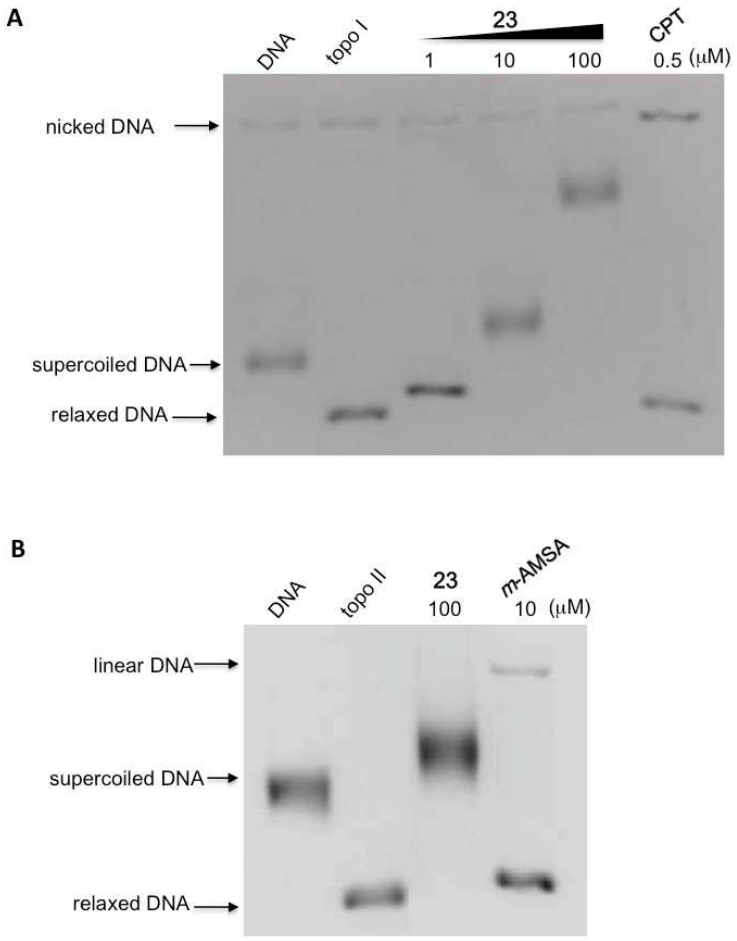


Figure 6. Effect of **23** on the stabilization of covalent DNA-topoisomerase I (A) and DNA-topoisomerase II (B) complex.

3. Conclusions

Some novel pyrrolo[3,2-*f*]quinazolinones (compounds **4**-**12**) and pyridoquinazolinones (compounds **15, 16**, **22** and **23**) were synthesized following different multi-step pathways. Intermediates and final compounds were fully characterized by instrumental techniques, in particular the exact structure of the angular pyridoquinazolinone **16** was achieved by HRMS and 1D and 2D NMR experiments. The latter allowed observation of the HMBC and NOE correlations for compound **16** and by this way to confirm the unexpected structure shown in figure 2. Worthy to note is that compound **23** was effortlessly obtained thanks to the selective hydrolytic reaction of the intermediate quinazolinone **19a** in mixture with **19b**, giving the corresponding easy isolable acid **20.**

The investigation on the antiproliferative effect highlighted the ability of the linear pyrido[2,3-*g*]quinazolinone **23** to induce a significant growth inhibition on all tested cell lines, with GI_{50} values in the low- or sub-micromolar range. Linear flow dichroism experiments demonstrated the capacity

of the planar chromophore to insert between DNA base pairs. The investigation of the effect of **23** on topoisomerase I and II catalytic activity showed a notable inhibition of the relaxation ability of supercoiled DNA mediated by the two nuclear enzymes. These results suggest that the effective intercalative complexation between **23** and DNA could perturb the macromolecule conformation, thus interfering with topoisomerase catalytic activity.

4. Material and methods

4.1 General experimental conditions

Melting points were determined on a Buchi M-560 capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a PerkinElmer 1760 FTIR spectrometer with potassium bromide pressed disks and on a Varian ATR FTIR; all values are expressed in cm^{-1}. UV−Vis spectra were recorded on a Thermo Helyos α spectrometer. ^1H NMR spectra were determined on Bruker 300 and 400 MHz spectrometers, with the solvents indicated; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. Coupling constants are given in Hertz. Integrals were satisfactorily in line with those expected on the basis of the compound structure. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, on a PerkinElmer C, H, N elemental analyzer model 240B, and analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Analytical data are presented in detail for each final compound in the Supplementary Data. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and on an Applied Biosystems Mariner System 5220 LC/MS (nozzle potential 140 eV). Column flash chromatography was performed on Merck silica gel (250−400 mesh ASTM); chemical reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F-254 glass plates. Solutions were concentrated on a rotary evaporator under reduced pressure. Starting materials were purchased from Sigma-Aldrich and Alfa Aesar, and solvents were purchased from Carlo Erba, Fluka and Lab-Scan. DMSO was obtained anhydrous by distillation under vacuum and stored on molecular sieves.

The purity of new tested compounds was checked by HPLC on a VARIAN ProStar model 210, with detector DAD VARIAN ProStar 335. The analysis was performed with a flow of 1 mL/min, using a C-18 column (250 mm × 4.6 mm), a particle size of 5 μm, and a loop of 10 μL. The detector was set at 300 nm. The mobile phase consisted of phase A (Milli-Q H_2O, 18.0 MΩ, TFA 0.05%) and phase B (95% MeCN, 5% phase A). The gradient elution was performed as reported: 0 min, % B = 10; 0−20 min, % B = 90; 25 min, % B = 90; 26 min, % B = 10; 31 min, % B = 10.

4.1.1 General procedure for the synthesis of pyrroloquinazolinones (**4**−**8**).

In a 100 mL flask, the appropriate amino-indole derivative **1**-**3**[16] (0.30 g, 1.87 mmol) dissolved in DMF (30 mL) was added with ethoxycarbonylisothiocyanate (0.21 mL, 1.87 mmol) and the mixture was stirred at RT for 1 h. The progress of the reaction was checked by TLC (EtOAc/*n*-Hex, 8:2) until disappearance of the starting material and formation of the first intermediate. At the end, the mixture was cooled at 0 °C and added to dimethylamine (0.28 mL, 5.61 mmol) and $HgCl_2$ (0.5 g, 1.87 mmol) and was left to stir at RT overnight. At the end, after that the formation of the second intermediate was completed (TLC, EtOAc/*n*-Hex, 8:2), the mixture was heated under reflux (160 °C) for about 2 hours until the previously mentioned intermediate disappeared with the concomitant formation of a new fluorescent spot corresponding to the final compound (TLC, $CHCl_3$/MeOH, 9:1) was evident. The reaction took about 40 hours. Then the mixture was cooled, filtered through a celite pad. The filtrate was evaporated under vacuum to dryness and the obtained solid was recrystallized from acetonitrile.

4.1.1.1 3-(Dimethylamino)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (4)

Grey powder. Yield: 30%; mp: 290 °C (decomposition); [Found: C, 62.9; H, 5.28; N, 24.45. $C_{12}H_{12}N_4O$ requires C, 63.2; H, 5.30; N, 24.6%]; R_f 0.29 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3400-2800 (br), 1691, 1470, 1390, 1255, 1105 cm^{-1}; δ_H (400 MHz, DMSO-d_6) 3.29 (s, 6H, N(C<u>H</u>$_3$)$_2$), 7.16 (dd, 1H, J 2.7 and 2.1 Hz, H-9), 7.62 (d, 1H, J 2.7 Hz, H-8), 7.64 (d, 1H, J 8.7 Hz, H-5), 7.92 (d, 1H, J 8.7 Hz, H-6), 11.86 (s, 1H, N<u>H</u>), 11.90 ppm (bs, 1H, CON<u>H</u>); δ_C (101 MHz, DMSO-d_6) 39.72 (N(<u>C</u>H$_3$)$_2$), 102.69 (C-9), 105.84 (C-9b), 111.21 (C-9a), 120.58 (C-5), 123.86 (C-6), 129.23 (C-6a), 133.05 (C-8), 149.78 (C-4a), 150.15 (C-3), 161.50 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{12}H_{13}N_4O^+$ requires 229.1084; found, 229.1078; RP-C18 HPLC: t_R 11.7 min, 97.5%.

4.1.1.2 3-(4-Methylpiperazin-1-yl)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (5)

Grey solid. Yield: 25%; mp: ≥290 °C (decomposition); [Found: C, 63.6; H, 6.05; N, 24.70. $C_{15}H_{17}N_5O$ requires C, 63.40; H, 6.03; N, 24.65%]; R_f 0.22 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3410-2850 (br), 1690, 1475, 1392, 1260, 1110 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 2.78 (s, 3H, NC<u>H</u>$_3$), 3.10-2.98 (m, 4H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_2$NCH$_3$), 4.30-4.17 (m, 4H, N(C<u>H</u>$_2$)$_2$(CH$_2$)$_2$NCH$_3$), 7.11 (d, 1H, J 8.6 Hz, H-5), 7.15 (dd, 1H, *J* 3.07 and 2.01 Hz, H-9), 7.51 (dd, 1H, *J* 8.6 and 2.1 Hz, H-6), 7.81 (dd, 1H, *J* 3.02 and 0.7 Hz, H-8), 11.47 (s, 1H, N<u>H</u>), 11.53 ppm (s, 1H, CON<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 42.73 (N(CH$_2$)$_2$(<u>C</u>H$_2$)$_2$NCH$_3$), 42.89 (N(<u>C</u>H$_2$)$_2$(CH$_2$)$_2$NCH$_3$), 52.09 (N<u>C</u>H$_3$), 103.14 (C-9), 109.01 (C-9b), 119.56 (C-9a), 123.83 (C-5), 126.45 (C-6), 127.82 (C-8), 132.52 (C-

6a), 146.07 (C-3), 149.10 (C-4a), 163.43 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{15}H_{18}N_5O^+$ requires 284.1506; found, 284.1510; RP-C18 HPLC: t_R 14.7 min, 99.7%.

4.1.1.3 3-(Dimethylamino)-7-ethyl-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (6)

Beige solid. Yield: 25%; mp: 250 °C (decomposition); [Found: C, 65.40; H, 6.27; N, 21.80. $C_{14}H_{16}N_4O$ requires C, 65.6; H, 6.29; N, 21.9%]; R_f 0.62 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3450-3330 (br), 1690, 1475, 1392, 1260, 1110 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.36 (t, *J* 7.3 Hz, 3H, $CH_2C\underline{H}_3$), 2.87 (s, 6H, $N(C\underline{H}_3)_2$), 4.26 (q, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_3$), 7.12 (d, *J* 2.9 Hz, 1H, H-9), 7.20 (d, *J* 8.8 Hz, 1H, H-5), 7.50 (d, *J* 2.9 Hz, 1H, H-8), 7.80 (d, *J* 8.8 Hz, 1H, H-6), 11.33 ppm (s, 1H, CON<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 16.30 ($CH_2\underline{C}H_3$), 38.71 ($N(\underline{C}H_3)_2$), 40.90 ($\underline{C}H_2CH_3$), 102.30 (C-9), 106.99 (C-9b), 114.71 (C-9a), 118.16 (C-6), 120.08 (C-5), 124.39 (C-8), 130.45 (C-6a), 145.24 (C-3), 148.88 (C-4a), 162.91 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{14}H_{17}N_4O^+$ requires 257.1397; found, 257.1395; RP-C18 HPLC: t_R 16.45 min, 98.5%.

4.1.1.4 7-Ethyl-3-(4-methylpiperazin-1-yl)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (7)

Brown solid. Yield: 57%; mp: 280 °C (decomposition); [Found: C, 65.40; H, 6.78; N, 22.43. $C_{17}H_{21}N_5O$ requires C, 65.6; H, 6.80; N, 22.50%]; R_f 0.31 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3450-3330 (br), 1688, 1474, 1387, 1254, 1109 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.37 (t, *J* 7.3 Hz, 3H, $CH_2C\underline{H}_3$), 2.78 (s, 3H, $NC\underline{H}_3$), 3.10-2.86 (m, 4H, $N(CH_2)_2(C\underline{H}_2)_2NCH_3$), 4.42-4.30 (m, 4H, $N(C\underline{H}_2)_2(CH_2)_2NCH_3$), 4.27 (q, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_3$), 7.11 (d, *J* 8.6 Hz, 1H, H-5), 7.14 (dd, *J* 3.0 Hz and J 0.74 Hz, 1H, H-9), 7.51 (d, *J* 2.9 Hz, 1H, H-8), 7.85 (d, *J* 9.2 Hz, 1H, H-6), 11.53 ppm (s, 1H, CON<u>H</u>); δ_C (101 MHz, DMSO-d_6) 16.19 ($CH_2\underline{C}H_3$), 40.82 ($\underline{C}H_2CH_3$), 42.84 ($N(CH_2)_2(\underline{C}H_2)_2NCH_3$), 43.01 ($N(\underline{C}H_2)_2(CH_2)_2NCH_3$), 52.18 ($N\underline{C}H_3$), 102.33 (C-9), 108.47 (C-9b), 119.12 (C-9a), 124.49 (C-5), 125.83 (C-6), 129.43 (C-8), 131.91 (C-6a), 146.22 (3-C), 149.72 (4a-C), 163.43 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{17}H_{22}N_5O^+$ requires 312.1819; found, 312.1802 and 156.5916; RP-C18 HPLC: t_R 16.47 min, 97.4%.

4.1.1.5 3-(Dimethylamino)-7-ethyl-2,7-dihydro-1-oxo-1*H*-pyrrolo[3,2-*f*]quinazoline-9-carbonitrile (8)

Slight pink solid. Yield 35%; mp: 260-264 °C; [Found: C, 63.9; H, 5.36; N, 24.85. $C_{15}H_{15}N_5O$ requires C, 64; H, 5.37; N, 24.90%]; R_f 0.46 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3460-3320 (br), 2240-2215, 1692, 1463, 1374, 1250, 1110 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.40 (t, *J* 7.3 Hz, 3H, $CH_2C\underline{H}_3$), 3.33 (s, 6H, $N(C\underline{H}_3)_2$), 4.31 (q, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_3$), 7.21 (d, *J* 8.9 Hz, 1H, H-5), 7.90 (d, *J* 8.9 Hz, 1H, H-6), 8.32 (s, 1H, H-8), 11.44 ppm (s, 1H, CON<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 15.66 ($CH_2\underline{C}H_3$), 37.71 ($N(\underline{C}H_3)_2$), 41.63 ($\underline{C}H_2CH_3$), 86.31 (C-9), 110.01 (<u>C</u>N), 117.62

(C-9b), 118.59 (C-9a), 120.35 (C-6), 121.90 (C-5), 131.10 (C-6a), 138.61 (C-8), 149.18 (C-4a), 151.28 (C-3), 162.82 ppm (C-1); HRMS (ESI-MS, 140 eV): *m/z* [M+H$^+$] $C_{15}H_{16}N_5O^+$ requires 282.1349; found, 282.1342; RP-C18 HPLC: t_R 13.95 min, 96.5%.

4.1.1.6 3-(Dimethylamino)-7-ethyl-2,7-dihydro-1-oxo-1H-pyrrolo[3,2-f]quinazoline-9-carbaldehyde (9)

As a common procedure, in a 2-neck 100 mL flask, $POCl_3$ (1.5 mL) was introduced slowly to DMF (3 mL), keeping the temperature around 10˚C by means of a water/ice bath. After about 15 min, a mixture of pyrroloquinazolinone **6** (0.30 g, 1.17 mmol) and DMF (2 mL) was added portion wise, maintaining the temperature below 10 °C. The reaction mixture was then heated at 50 °C. for about 3 h while the progress was checked by TLC ($CHCl_3$/MeOH, 9:1). At the end of the reaction the mixture was cooled to RT and then added with water and ice before making it alkaline with 10% NaOH. The formed final suspension was filtered and the filtrate was extracted with EtOAc. The combined extracts were evaporated to dryness under vacuum giving a sandy coloured solid. Yield 87%; mp: 260 °C (decomposition); [Found: C, 63.20; H, 5.66; N, 19.67. $C_{15}H_{16}N_4O_2$ requires C, 63.4; H, 5.67; N, 19.7%]; R_f 0.53 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3470-3330 (br), 2830-2810, 2750-2720, 1765-1645, 1470, 1390, 1235, 1115 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.41 (t, *J* 7.3 Hz, 3H, $CH_2C\underline{H}_3$), 3.12 (s, 6H, $N(C\underline{H}_3)_2$), 4.34 (q, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_3$), 7.24 (d, *J* 8.8 Hz, 1H, H-6), 7.92 (d, *J* 8.9 Hz, 1H, H-5), 8.19 (s, 1H, H-8), 11.05 (s, 1H, C<u>H</u>O), 11.15 ppm (s, 1H, CON<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 15.74 ($CH_2\underline{C}H_3$), 37.58 ($N(\underline{C}H_3)_2$), 41.71 ($\underline{C}H_2CH_3$), 102.06 (C-9), 109.44 (C-9b), 118.90 (C-6), 120.04 (C-9a), 121.48 (C-5), 132.49 (C-6a), 133.89 (C-8), 147.23 (C-3), 149.01 (C-4a), 162.99 (C-1), 191.79 ppm (<u>C</u>HO); HRMS (ESI-MS, 140 eV): *m/z* [M+H$^+$] $C_{15}H_{17}N_4O_2^+$ requires 285.1346; found, 285.1341; RP-C18 HPLC: t_R 12.38 min, 97.4%.

4.1.1.7 7-Ethyl-3-(4-methylpiperazin-1-yl)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one-9-carbaldehyde (10)

Compound **10** was obtained as reported for compound **9**, by reacting 1 g of compound **7** and obtaining 0.563 g of a pale yellow powdery solid. Yield: 52%; mp: 290 °C (decomposition); [Found: C, 63.6; H, 6.23; N, 20.6. $C_{18}H_{21}N_5O_2$ requires C, 63.7; H, 6.24; N, 20.6%]; R_f 0.24 ($CHCl_3$/MeOH, 9:1; ν_{max} (ATR, ZnSe) 3465-3320 (br), 2828, 2735, 1724, 1481, 1388, 1223, 1112 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.40 (t, *J* 7.3 Hz, 3H, $CH_2C\underline{H}_3$), 2.20 (s, 3H, $NC\underline{H}_3$), 2.55-2.39 (m, 4H, $N(CH_2)_2(C\underline{H}_2)_2NCH_3$), 3.62-3.46 (m, 4H, $N(C\underline{H}_2)_2(CH_2)_2NCH_3$), 4.35 (q, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_3$), 7.23 (d, *J* 9.2 Hz, 1H, H-5), 7.96 (d, *J* 8.9 Hz, 1H, H-6), 8.23 (s, 1H, H-8), 11.15 (s, 1H, C<u>H</u>O), 11.49 ppm (s, 1H, CON<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 15.75 ($CH_2\underline{C}H_3$), 41.68 ($\underline{C}H_2CH_3$), 42.92 ($N(CH_2)_2(\underline{C}H_2)_2NCH_3$), 44.12 ($N(\underline{C}H_2)_2(CH_2)_2NCH_3$), 53.34 ($N\underline{C}H_3$), 110.40 (C-

9), 111.01 (C-9b), 115.93 (C-9a), 118.89 (C-6), 121.51 (C-5), 133.03 (C-6a), 134.38 (C-8), 145.84 (C-4a), 149.65 (C-3), 161.19 (C-1), 191.51 ppm (CHO); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{18}H_{22}N_5O_2^+$ requires 340.1768; found, 340.1721 and 170.5959; RP-C18 HPLC: t_R 15.50 min, 98.0%.

4.1.2 General Procedure for the Synthesis of 9-Methyl-Alcohol-Substituted Pyrroloquinazoline Derivatives **11**, **12**. In a 250 mL flask, on the basis of the starting material, a double molar amount of $NaBH_4$ was suspended in MeOH (5 mL). The ethylpyrroloquinazoline 9-carbaldehyde derivative **9**, **10** (3−5 mmol) dissolved in of MeOH (10 mL) was slowly added under inert atmosphere (N_2) and at room temperature. The course of the reaction was monitored by TLC, in all cases noting the appearance of a spot with a R_f lower than that of the starting 9-carbaldehyde compound. At the end of the reaction lasting about 3 h, the excess of $NaBH_4$ was quenched with a saturated aqueous solution of NH_4Cl and the solid was separated by filtration and washed with EtOAc some times. The total filtrate was dried with anhydrous Na_2SO_4 and evaporated under vacuum, yielding the crude reaction product which was purified by liquid chromatography.

4.1.2.1 3-(Dimethylamino)-7-ethyl-9-(hydroxymethyl)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (11)

The general procedure was followed by adding dropwise a solution of the 9-carbaldehyde derivative **9** (2.445 g, 3.77 mmol) in MeOH (10 mL) to a suspension of $NaBH_4$ (0.539 g,7.54 mmol) in MeOH (5 mL). At the end of the reaction (2 h), by workup of the reaction mixture, a crude yellow-orange solid was obtained (1.024 g). After purification in a chromatographic column (eluent $CHCl_3$/MeOH, 9:1) a yellow solid corresponding to compound **11** was obtained (0.953 g). Yield: 92.2%; mp: 244 °C (decomposition); [Found: C, 62.8; H, 6.33; N, 19.53. $C_{15}H_{18}N_4O_2$ requires C, 62.9; H, 6.34; N, 19.6%]; R_f 0.84 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3660-3250 (br), 1645, 1430, 1388, 1075, 1050 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.43 (t, *J* 7.25 Hz, 3H, CH_2CH_3), 3.24 (s, 6H, $N(CH_3)_2$), 4.38 (q, *J* 7.26 Hz, 2H, CH_2CH_3), 5.10 ppm (d, *J* 5.31 Hz, 2H, -CH_2OH); 5.59 (t, *J* 5.31 Hz, 1H, -OH), 7.25 (d, *J* 9.16 Hz, 1H, H-6), 7.88 (d, *J* 9.04 Hz, 1H, H-5), 7.99 (s, 1H, H-8), 11.39 ppm (s, 1H, CONH); δ_C NMR (101 MHz, DMSO-d_6) 16.08 (CH_2CH_3), 38.34 ($N(CH_3)_2$), 42.04 (CH_2CH_3), 60.98 (CH_2OH), 104.44 (C-9), 110.12 (C-9b), 119.45 (C-6), 121.73 (C-9a), 122.83 (C-5), 133.94 (C-6a), 140.12 (C-8), 148.01 (C-3), 149.23 (C-4a), 163.44 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{15}H_{19}N_4O_2^+$ requires 287.1503; found, 287.1513; RP-C18 HPLC: t_R 10.52 min, 98.5%.

4.1.2.2 3-(4-methylpiperazin-1-yl)-7-ethyl-9-(hydroxymethyl)-2*H*-pyrrolo[3,2-*f*]quinazolin-1(7*H*)-one (12)

Following the general procedure, to a suspension of $NaBH_4$ (0.275 g, 7.23 mmol) in MeOH (5 mL) was slowly added the 9-carbaldehyde derivative **10** (1.253 g, 3.62 mmol) in MeOH (10 mL). At the end of the reaction (2 h), on TLC a yellow spot having a R_f lower than that of the starting compound. By workup of the reaction mixture a crude yellow-beige solid residue was obtained (1.050 g), which was purified on chromatographic column ($CHCl_3$/MeOH, 9:1), yielding compound **12** as a beige solid residue (0.950 g). Yield: 86.5%; mp: 275 °C (decomposition); [Found: C, 63.2; H, 6.78; N, 20.47. $C_{18}H_{23}N_5O_2$ requires C, 63.3; H, 6.79; N, 20.5%]; R_f 0.89 ($CHCl_3$/MeOH, 9:1); ν_{max} (ATR, ZnSe) 3650-3200, 1680, 1415, 1400, 1112, 987 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.45 (t, *J* 7.47 Hz, 3H, $CH_2C\underline{H}_3$), 2.24 (s, 3H, $NC\underline{H}_3$), 2.65-2.44 (m, 4H, $N(CH_2)_2(C\underline{H}_2)_2NCH_3$), 3.42-3.28 (m, 4H, $N(C\underline{H}_2)_2(CH_2)_2NCH_3$), 4.42 (q, *J* 7.42 Hz, 2H, $C\underline{H}_2CH_3$), 5.09 (d, *J* 5.51 Hz, 2H, -$C\underline{H}_2$OH), 5.57 (t, *J* 5.51 Hz, 1H, -O$\underline{H}$), 7.34 (d, *J* 9.07 Hz, 1H, H-5), 7.92 (d, *J* 9.03 Hz 1H, H-6), 8.04 (s, 1H, H-8), 11.54 ppm (s, 1H, CON$\underline{H}$); δ_C NMR (101 MHz, DMSO-d_6) 16.01 ($CH_2\underline{C}H_3$), 42.04 ($\underline{C}H_2CH_3$), 42.88 ($N(CH_2)_2(\underline{C}H_2)_2NCH_3$), 45.02 ($N(\underline{C}H_2)_2(CH_2)_2NCH_3$), 54.11 ($N\underline{C}H_3$), 60.45 ($\underline{C}H_2$OH) 111.43 (C-9), 115.23 (C-9b), 120.04 (C-9a), 122.23 (C-6), 125.43 (C-5), 132.98 (C-6a), 134.90 (C-8), 145.23 (C-4a), 150.03 (C-3), 163.87 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{18}H_{24}N_5O_2^+$ requires 342.1925; found, 342.1921 and 171.5994; RP-C18 HPLC: t_R 10.99 min, 96.8%.

4.1.3 6,6-Dimethyl-3-{[(4-oxo-3,4-dihydroquinazolin-6-yl)amino]methylidene}oxane-2,4-dione (14)

A solution of Meldrum's acid (1.427 g, 9.91 mmol) in an excess of methyl orthoformate (15 mL) was heated to reflux for two hours under nitrogen atmosphere. The solution was then cooled to room temperature and the starting 6-aminoquinazolinone (**13**, 1.228 g, 7.62 mmol) was added portion wise. The mixture thus obtained was maintained at reflux for 6 hours, checking the progress of the reaction by TLC (eluent $CHCl_3$/MeOH, 8:2). The reaction mixture was partly evaporated in a rotavapor, then taken up with water and subsequently extracted with EtOAc. The combined extracts were dried with anhydrous sodium sulfate, filtered and then evaporated to dryness. The crude reaction residue was purified by silica gel column chromatography (eluent $CHCl_3$/MeOH, 8:2) and additionally re-crystallized from MeOH yielding 2.063 g (6.54 mmol) of a yellow powdery solid. Yield: 85.8%; mp: 247 °C; R_f 0.52 ($CHCl_3$/MeOH, 8:2); ν_{max} (ATR, ZnSe) 3421-3108, 1750, 1740, 1675, 1415, 1400, 1112, 987 cm^{-1};δ_H NMR (400 MHz, DMSO-d_6) 1.69 (s, 6H, $C(C\underline{H}_3)_2$), 7.72 (d, *J* 8.83, 1H, H-8), 8.04 (dd, *J* 8.83 Hz and J 2.70 Hz, 1H, H-7), 8.09 (d, *J* 3.26 Hz, 1H, H-2), 8.19 (d, *J* 2.63 Hz, 1H, H-5), 8.62 (d, *J* 14.74 Hz, 1H, =C$\underline{H}$NH), 11.40 (d, *J* 14.46 Hz, 1H, =CHN$\underline{H}$), 12.34

ppm (bs, 1H, CONH); δ_C NMR (101 MHz, DMSO-d_6) 26.86 (C($\underline{C}H_3)_2$), 87.72 (=$\underline{C}$HNH), 104.54 ($\underline{C}(CH_3)_2$), 115.54 (C-5), 123.63 (C-4a), 126.39 (C-7), 129.05 (C-8), 145.83 (C-2), 147.10 (C-8a), 159.92 (C-4), 163.15 (C=O), 163.96 (C=O), 167.65 (C-4), 197.58 ppm ($\underline{C}$=CH); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{15}H_{14}N_3O_5^+$ requires 316.0933; found, 316.0930.

4.1.4 10-Hydroxy-1*H*,2*H*,7*H*,10*H*-pyrido[3,2-*f*]quinazolin-1-one (15)

Boiling Ph_2O (~10 mL) was quickly added to a flask containing the quinazolinone derivative **14** (2.063 g, 6.54 mmol). The reaction mixture was maintained at reflux for 15min and then cooled to room temperature. Subsequently, Et_2O (20mL) was added to the mixture and the formation of a precipitate was observed which was allowed to deposit at room temperature overnight. It was then collected, washed several times with Et_2O and dried in a vacuum oven, resulting in 1.302 g of a brownish powdery solid. Yield: 93.0%; mp > 330 °C (decomposition); [Found: C, 61.8; H, 3.30; N, 19.67. $C_{11}H_7N_3O_2$ requires C, 62; H, 3.31; N, 19.7%]; R_f 0.47 (blue fluorescent spot, $CHCl_3$/MeOH, 8:2); ν_{max} (ATR, ZnSe) 3455-3100, 1702, 1680, 1447, 1394, 1117, 999 cm^{-1}; HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{11}H_8N_3O_2^+$ requires 214.0617; found, 214.0625.

4.1.5 2-[2-(Diethylamino)ethyl]-10-hydroxy-1*H*,2*H*,7*H*,10*H*-pyrido[3,2-*f*]quinazolin-1-one hydrochloride (16)

In a dried 100 mL flask, excess NaH (60 % dispersion in mineral oil, 0.680 g, 2.82 mmol) was added and washed three times with toluene. Via a funnel, compound **15** (0.200 g, 0.94 mmol) suspended in anhydrous DMF (5 mL) was added dropwise, and the mixture changed from light brown to deep brown with concomitant development of hydrogen fumes. The reaction mixture was left stirring for 1 h at room temperature. Then, the halo-compound 2-chloro-*N*,*N*-diethylethanamine(threefold molar excess over starting compound, 0.485 g, 2.82 mmol) and triethylamine (0.39 mL, 2.82 mmol, 0.728 g/mL) both dissolved in anhydrous DMF (1 mL) were added, and the solution was left stirring at room temperature for 5 h, monitoring reaction progress by TLC ($CHCl_3$/MeOH, 8:2). At the end, H_2O (25 mL) was added to the reaction mixture, which was then extracted with EtOAc. The combined extracts were washed several times with H_2O, brine, dried over anhydrous $MgSO_4$, filtered and finally evaporated to dryness by rotary evaporation, obtaining a yellow semisolid residue which was then triturated with cyclohexane. Finally, the powdery solid was dissolved in an EtOAc/absolute EtOH, 2:1 mixture, and dry HCl gas was bubbled into the solution until a yellowish precipitate formed. The mixture was left at 0−4 °C overnight, and the resulting precipitate was collected and dried under vacuum to yield 0.125 g of a powdery gold solid. Yield: 43%; mp: 268 °C; [Found: C, 58.4; H, 6.06; N, 16.02. $C_{17}H_{20}N_4O_2$ requires C, 58.5; H, 6.07; N, 16.1%]; R_f 0.54 (blue fluorescent spot, $CHCl_3$/MeOH, 8:2); ν_{max} (ATR,

ZnSe) 3455-3100, 2700-2250 (br), 1704, 1688, 1450, 1390, 1116, 998 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 0.81 (t, *J* 7.08 Hz, 6H, $CH_2C\underline{H_3}$), 2.49 (q, *J* 7.24 Hz, 4H, $C\underline{H_2}CH_3$), 2.75 (t, *J* 5.87 Hz, 2H, $NCH_2C\underline{H_2}N$); 4.23 (t, *J* 5.68 Hz, 2H, $NC\underline{H_2}CH_2N$), 7.02 (d, *J* 4.50 Hz, 1H, H-9), 7.92 (d, *J* 9.20 Hz, 1H, H-5), 8.16 (bs, 1H, N<u>H</u>Cl), 8.30 (d, *J* 9.01 Hz, 1H, H-6), 8.66 (d, *J* 4.50 Hz, 1H, H-8), 8.67 (s, 1H, H-3), 15.11 ppm (C(10)-O<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 12.44 ($CH_2\underline{C}H_3$), 46.18 ($N\underline{C}H_2CH_2N$), 47.10 ($\underline{C}H_2CH_3$), 50.38 ($NCH_2\underline{C}H_2N$), 111.37 (C-9), 116.15 (C-10b), 117.77 (C-10a), 130.37 (C-5), 139.41 (C-6), 149.22 (C-3), 150.50 (C-4a), 151.57 (C-6a), 152.01 (C-8), 163.39 (C-10), 164.15 ppm (C-1); HRMS (ESI-MS, 140 eV): *m*/*z* [$M+H^+$] $C_{17}H_{21}N_4O_2^+$ requires 313.1759; found, 313.1710 and 157.0925; RP-C18 HPLC: t_R 4.89 min, 96.2%.

4.1.6 Ethyl 5-amino-2-nitrobenzoate (17)

In a 250 mL round-bottomed flask, commercial 5-amino-2-nitrobenzoic acid (1.088 g, 5.97 mmol) was dissolved in absolute EtOH (100 mL). The mixture was then saturated with dry HCl gas, refluxed and stirred for 6 h. After 6 h all the starting reagent had disappeared, so EtOH was removed under reduced pressure. The resulting residue was treated with sat'd $NaHCO_3$ and extracted with EtOAc (2×20 mL). The organic extracts were washed with water, brine, dried over Na_2SO_4 and finally concentrated to give 1.129 g (5.37 mmol) of a pure brown powdery solid. Yield: 89%; R_f 0.75 ($CHCl_3$/MeOH, 8:2); mp: 105 °C; δ_H NMR (400 MHz, DMSO-d_6) 1.25 (t, *J* 7.06 Hz, 3H, $CH_2C\underline{H_3}$), 4.28 (q, *J* 7.04 Hz, 2H, $C\underline{H_2}CH_3$), 6.60 (d, *J* 2.46 Hz, 1H, H-5), 6.67 (dd, *J* 9.10 Hz and *J* 2.48 Hz, 1H, H-4), 6.90 (bs, 2H, -$N\underline{H_2}$), 7.91 ppm (d, *J* 9.08, 1H, H-3); δ_C NMR (101 MHz, DMSO-d_6) 13.64 ($CH_2\underline{C}H_3$), 61.44 ($\underline{C}H_2CH_3$), 111.37 (C-4), 113.11 (C-5), 127.41 (C-3), 132.44 (C-2), 154.81 (C-5), 166.65 ppm (<u>C</u>OO).

4.1.7 Ethyl 5-{[(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylidene)methyl]amino}-2-nitrobenzoate (18)

A solution of Meldrum's acid (1.006 g, 6.98 mmol) in an excess of methyl orthoformate (15 mL) was heated to reflux for two hours under nitrogen atmosphere. The solution was then cooled to room temperature and the starting ethyl 5-amino-2-nitrobenzoate (**17**, 1.129 g, 5.37 mmol) was added portion wise. The mixture thus obtained was maintained at reflux for 6 hours, checking the progress of the reaction by TLC (eluent $CHCl_3$/MeOH, 8:2). The reaction mixture was partly evaporated in a rotavapor, then taken up with water and subsequently extracted with EtOAc. The combined extracts were dried with anhydrous sodium sulfate, filtered and then evaporated to dryness. The crude reaction residue was purified by silica gel column chromatography (eluent $CHCl_3$/MeOH, 9:1) and additionally re-crystallized from MeOH yielding 1.011 g (2.77 mmol) of a crystalline light brown powdery solid. Yield: 52.0%; mp: 221 °C R_f 0.79 ($CHCl_3$/MeOH, 9:1); ν_{max}

(ATR, ZnSe) 3108, 2987, 1748, 1731, 1648, 1490, 1315, 870, 706 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 1.31 (t, *J* 7.09 Hz, 3H, $CH_2C\underline{H}_3$), 1.70 (s, 6H, $C(C\underline{H}_3)_2$, 4.35 (q, *J* 7.10, 2H, $C\underline{H}_2CH_3$), 7.96 (dd, *J* 8.94 Hz and *J* 2.51 Hz, 1H, H-4), 8.09 (d, *J* 2.53 Hz, 1H, H-6), 8.17 (d, *J* 8.92 Hz, 1H, H-3), 8.69 (d, *J* 14.34 Hz, 1H, C=C<u>H</u>), 11.39 ppm (d, *J* 14.36 Hz, 1H, N<u>H</u>); δ_C NMR (101 MHz, DMSO-d_6) 14.16 ($CH_2\underline{C}H_3$), 27.03 ($C(\underline{C}H_3)_2$), 62.74 ($\underline{C}H_2CH_3$), 89.75 ($\underline{C}(CH_3)_2$), 104.94 (C=<u>C</u>H), 120.02 (C-6), 122.12 (C-4), 126.56 (C-3), 129.70 (C-1), 143.75 (C-5), 143.85 (C-2), 153.94 (<u>C</u>=CH), 162.88 (CO), 163.57 (CO), 165.03 ppm (COO); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{16}H_{17}N_2O_8^+$ requires 365.0985; found, 365.0982.

4.1.8 Ethyl 6-nitro-4-oxo-1,4-dihydroquinoline-7-carboxylate (19a) and ethyl 6-nitro-4-oxo-1,4-dihydroquinoline-5-carboxylate (19b)

Boiling Ph_2O (~10 mL) was quickly added to a flask containing derivative **18** (0.505 g, 1.39 mmol). The reaction mixture was maintained at reflux for 15 min and then cooled to room temperature. Subsequently, Et_2O (20mL) was added to the mixture and the formation of a precipitate was observed which was allowed to deposit at room temperature overnight. It was then collected, washed several times with Et_2O and dried in a vacuum oven, resulting in 0.283 g of a brownish powdery solid constituted by an irresolvable mixture of **19a** and **19b**. Yield: 77.0%; R_f 0.63 and 0.61 ($CHCl_3$/MeOH, 8:2); mp: 134 °C; δ_H NMR (400 MHz, DMSO-d_6) 1.30-1.16 (m, 6H, $CH_2C\underline{H}_3$), 4.36 (q, *J* 7.08 Hz, 4H, $C\underline{H}_2CH_3$), 6.14 (d, *J* 7.55 Hz, 1H, H-3, 19b), 6.23 (d, *J* 7.57 Hz, 1H, H-3, 19a), 7.77 (d, 1H, *J* 9.27 Hz, H-7, 19b), 7.88 (s, 1H, H-8, 19a), 8.03 (d, *J* 7.46 Hz, 1H, H-2, 19b), 8.10 (d, *J* 7.63 Hz, 1H, H-2, 19a), 8.40 (d, *J* 9.32 Hz, 1H, H-8, 19b), 8.65 (s, 1H, H-5, 19a), 12.34 ppm (bs, 2H, N<u>H</u>). Yield determined by NMR molar ratio evaluation: 63%, **19a**; 37%, **19b**; HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{12}H_{11}N_2O_5^+$ requires 263.0668; found, 263.0660.

4.1.9 6-Nitro-4-oxo-1,4-dihydroquinoline-7-carboxylic acid (20)

In a 250 mL round-bottomed flask, the unresolvable mixture of isomers **19a** and **19b** (0.645 g, 2.46 mmol) was dissolved in MeOH and the resulting solution was cooled by mean of an ice bath. Approximately 10 mL of a NaOH 20% solution were consequently added and the mixture was allowed to stand at room temperature overnight. After 24 h only one of the TLC spot was completely disappeared, MeOH was removed by mean of a rotary evaporator and the resulting water remnants were extracted with EtOAc (3×20 mL). The combined organic fractions were dried with anhydrous sodium sulfate, filtered and then evaporated to dryness, leading to a yellow powdery product, corresponding to **19b** (0.270 g, 1.02 mmol). Yield: 41.4%; R_f 0.61 ($CHCl_3$/MeOH, 8:2); mp: 132.8; ν_{max} (ATR, ZnSe) 3420-3075, 2988, 1698, 1650, 1488, 1112, 980 cm^{-1}; °C; δ_H NMR (400 MHz, DMSO-d_6) 1.32 (t, *J* 7.27 Hz, 3H, $CH_2C\underline{H}_3$), 4.34 (q, *J* 7.24 Hz, 2H,

CH_2CH_3), 6.14 (d, *J* 7.44 Hz, 1H, H-3), 7.75 (d, 1H, *J* 9.04 Hz, H-7), 8.06 (d, *J* 7.45 Hz, 1H, H-2), 8.42 (d, *J* 9.10 Hz, 1H, H-8), 12.21 ppm (bs, 1H, NH); δ_C NMR (101 MHz, DMSO-d_6) 14.13 (CH_2CH_3), 61.18 (CH_2CH_3), 109.05 (C-3), 113.14 (C-5), 117.29 (C-7), 120.08 (C-5), 120.09 (C-8), 140.00 (C-2), 141.10 (C-8a), 148.37 (C-6), 164.50 (COOEt), 173.76 (C-4); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{12}H_{11}N_2O_5^+$ requires 263.0668; found, 263.0664. As the alkaline water fraction was made acidic (HCl 37%), a brown precipitate immediately formed. Collected by filtration on a G4 gooch filter, the resulting gummy solid was washed several times with water and finally dried in a vaccum oven overnight. 0.420 g (1.79 mmol) of a powdery deep brown solid compound **20** were obtained. Yield: 73%; mp: 311 °C; R_f 0.10 ($CHCl_3$/MeOH, 8:2); ν_{max} (ATR, ZnSe) 3550-2650, 1780, 1698, 1650, 1437, 1340, 1250 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 6.23 (d, *J* 7.53 Hz, 1H, H-3), 7.95 (s, 1H, H-8), 8.09 (d, 1H, *J* 7.50 Hz, H-2), 8.59 (s, 1H, H-5), 12.74 ppm (bs, 1H, NH); δ_C NMR (101 MHz, DMSO-d_6) 110.67 (C-3), 120.21 (C-8), 122.31 (C-5), 125.08 (C-4a), 131.17 (C-8a), 141.18 (C-2), 141.92 (C-7), 142.16 (C-6), 174.62 (C-4), 175.57 ppm (COOH); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{10}H_7N_2O_5^+$ requires 235.0355; found, 235.0349.

4.1.10 6-Amino-4-oxo-1,4-dihydroquinoline-7-carboxylic acid (21)

Into a three-necked flask of 500 mL, previously dried in an oven, about 0.300 g of C/Pd 10% and approximately 60 mL of EtOAcwere placed. After connecting the flask to an elastomer balloon containing hydrogen gas, the mixture was stirred at room temperature for 1 h in order to saturate the suspension of C/Pd with hydrogen. Next, derivative **20** (0.832 g, 3.17 mmol) in MeOH (250 mL) was added dropwise to the suspension, and the mixture was stirred under hydrogen at atmospheric pressure and heated by means of an oil bath at 50−60 °C for 15 h, monitoring the progress of the reaction by TLC analysis (*n*-Butanole/H_2O/AcOH, 3:1:1). At the end of the reaction, the mixture was filtered by mean of a celite pad, and the solution was concentrated to dryness on a rotavapor to give 0.609 g (2.98 mmol) of semisolid dark purple product. Yield: 94%; mp: 311 °C; R_f 0.58 (green fluorescent spot, *n*-Butanole/H_2O/AcOH, 3:1:1); ν_{max} (ATR, ZnSe) 3550-2650, 1777, 1699, 1635, 1605, 1440, 1102, 987 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 5.37 (bs, 2H, NH_2), 6.05 (d, *J* 6.99 Hz, 1H, H-3), 7.39 (s, 1H, H-8), 7.89 (d, *J* 6.93 Hz, 1H, H-2), 8.17 (s, 1H, H-8), 12.32 ppm (bs, 1H, NH); δ_C NMR (101 MHz, DMSO-d_6) 105.61 (C-3), 107.96 (C-4a), 116.25 (C-5), 122.18 (C-8), 130.01 (C-7), 139.07 (C-8a), 142.61 (C-2), 146.65 (C-6), 168.48 (COOH), 173.72 ppm (C-4); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H$^+$] $C_{10}H_9N_2O_3^+$ requires 205.0613; found, 205.0609.

4.1.11 3*H*,4*H*,6*H*,9*H*-Pyrido[2,3-*g*]quinazoline-4,9-dione (22)

A mixture of **21** (0.200g, 0.98 mmol), trimethylorthoformate (2 mL) and ammonium acetate (0.075 g, 0.98 mmol) was placed in a tall beaker. The beaker was covered with a stemless funnel and then

irradiated in a domestic microwave oven for 2 min with a power of 210 W. After a cooling time of about 5 min, the beaker and contents were irradiated at 250 W for an addition 5 min. The resultant residue was triturated with water and filtered off and finally purified by liquid column chromatography ($CHCl_3$/MeOH, 8:2). 0.028 g (0.13 mmol) of a pale brown solid were obtained. Yield: 13.2%; mp: 177 °C; [Found: C, 61.8; H, 3.30; N, 19.66. $C_{11}H_7N_3O_2$ requires C, 62; H, 3.31; N, 19.7%]; R_f 0.32 (blue fluorescent spot, $CHCl_3$/MeOH, 8:2); ν_{max} (ATR, ZnSe) 3400-2800 (br), 1745, 1691, 1488, 1382, 1263, 1112 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) 6.07 (d, *J* 7.38 Hz, 1H, H-8), 8.03 (d, *J* 7.44 Hz, 1H, H-7), 8.05 (s, 1H, H-2), 8.30 (s, 1H, H-10), 8.40 (s, 1H, H-5), 11.54 (bs, 1H, N(6)H), 12.40 ppm (bs, 1H, N(3)H); δ_C NMR (101 MHz, DMSO-d_6) 108.55 (C-8), 116.87 (C-5), 124.18 (C-10), 126.31 (C-4a), 130.87 (C-9a), 138.76 (C-5a), 141.89 (C-7), 143.72 (C-10a), 145.18 (C-2), 161.45 (C-4), 177.56 ppm (C-9); HRMS (ESI-MS, 140 eV): *m*/*z* [M+H^+] $C_{11}H_8N_3O_2^+$ requires 214.0616; found, 214.0617.

4.1.12 9-[2-(Diethylamino)ethoxy]-3-[2-(diethylamino)ethyl]-3*H*,4*H*-pyrido[2,3-*g*]quinazolin-4-one dihydrochloride (23)

In a dried 100 mL flask, excess NaH (60 % dispersion in mineral oil, 0.040 g, 1.40 mmol) was placed and washed three times with toluene. Via a funnel, compound **22** (0.100 g, 0.46 mmol) suspended in anhydrous DMF (1 mL) was added dropwise, and the mixture changed from light brown to deep brown with concomitant development of hydrogen fumes. The reaction mixture was left stirring for 1 h at room temperature. Then, the halo-compound 2-chloro-*N*,*N*-diethylethanamine(sixfold molar excess over starting compound, 0.484 g, 2.80 mmol) and triethylamine (0.39 mL, 2.80 mmol, 0.728 g/mL) both dissolved in anhydrous DMF (0.5 mL) were added, and the solution was left stirring at room temperature for 5 h, monitoring reaction progress by TLC ($CHCl_3$/MeOH, 8:2). At the end, H_2O (10 mL) was added to the reaction mixture, which was then extracted with EtOAc. The combined extracts were washed several times with H_2O, brine, dried over anhydrous $MgSO_4$, filtered and finally evaporated to dryness by rotary evaporation, obtaining a yellow semisolid residue which was then triturated with cyclohexane. Finally, the powdery solid was dissolved in an EtOAc/absolute EtOH, 2:1 mixture, and dry HCl gas was bubbled into the solution until a yellowish precipitate formed. The mixture was left at 0−4 °C overnight, and the resulting precipitate was collected and dried under vacuum to yield 0.105 g (0.21 mmol) of a powdery yellow solid. Yield: 46.8%; mp: 243 °C; [Found: C, 56.9; H, 7.26; N, 14.43. $C_{23}H_{34}N_5O_2$ requires C, 57; H, 7.28; N, 14.5%]; R_f 0.75 (blue fluorescent spot, $CHCl_3$/MeOH, 8:2); ν_{max} (ATR, ZnSe) 3425-2867 (br), 2750-2534 (br), 1685, 1478, 1334, 1276, 1124 cm^{-1}; δ_H NMR (400 MHz, DMSO-d_6) δ 0.85-0.98 (m, 12H, $CH_2C\underline{H_3}$), 2.51-2.71 (m, 8H, $C\underline{H_2}CH_3$), 2.86 (t, J 7.25

Hz, 2H, $OCH_2C\underline{H_2}N$), 2.91 (t, J 7.24 Hz, 2H, $NCH_2C\underline{H_2}N$), 4.11 (t, J 7.25 Hz, 2H, $NC\underline{H_2}CH_2N$), 4.30 (t, J 7.26 Hz, 2H, $OC\underline{H_2}CH_2N$), 6.96 (d, J 5.55 Hz, 1H, H-8), 8.10 (s, 1H, H-10), 8.37 (s, 1H, H-2), 8.42 (s, 1H, H-5), 8.72 ppm (d, J 5.50 Hz, 1H, H-7); δ_C NMR (101 MHz, DMSO-d_6) δ 11.62-11.74 ($CH_2\underline{C}H_3$), 47.12 ($NCH_2CH_2N(\underline{C}H_2CH_3)_2$), 47.69 ($OCH_2CH_2N(\underline{C}H_2CH_3)_2$), 50.89 ($N\underline{C}H_2CH_2N(CH_2CH_3)_2$), 52.94 ($NCH_2\underline{C}H_2N(CH_2CH_3)_2$), 53.83 ($OCH_2\underline{C}H_2N(CH_2CH_3)_2$), 68.16 ($O\underline{C}H_2CH_2N(CH_2CH_3)_2$), 110.72 (C-8), 118.72 (C-5), 125.24 (C-10), 126.37 (C-4a), 128.87 (C-9a), 140.82 (C-5a), 144.82 (C-7), 146.98 (C-10a), 147.72 (C-2), 163.56 (C-9), 165.18 ppm (C-4); HRMS (ESI-MS, 140 eV): *m*/2*z* [$M+2H^{2+}$] $C_{23}H_{35}N_5O_2^{2+}$ requires, 206.6390; found, 206.6384; RP-C18 HPLC: t_R 8.96 min, 99.5%.

4.2 Inhibition Growth Assay

HeLa (human cervix adenocarcinoma cells) were grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.); H460 (large cell lung carcinoma) and MSTO-211H (human biphasic mesothelioma) were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 2.38 g/L Hepes, 0.11 g/L pyruvate sodium and 2.5 g/L glucose. 1.5 g/L $NaHCO_3$, 10% heat-inactivated fetal calf serum (Invitrogen), 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. Cells (2.5-3 x 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 to the complete medium and incubated for a further 72 h. A Trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as GI_{50} values, i.e., the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

4.3. Nucleic acids

Salmon testes DNA was purchased from Sigma Chemical Company. The DNA concentration was determined using extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm. pBR322 DNA was purchased from Fermentas Life Sciences.

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 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, and each spectrum was accumulated twice.

Aqueous solutions of salmon testes DNA (1.9×10^{-3}M) in 10 mM ETN buffer (containing 0.01 M TRIS, 1 mM EDTA (pH 7.0) and 0.01 M NaCl) were used. Spectra were recorded at 25°C at [drug]/[DNA] = 0, 0.04 and 0.08.

4.5. DNA topoisomerase relaxation assay

Supercoiled pBR322 plasmid DNA (0.25 µg) was incubated with 1U topoisomerase II (human recombinant topoisomerase II α, USB Corporation) or 2U topoisomerase I (calf thymus topoisomerase I, Topogen) and the test compound as indicated for 60 min at 37°C in 20 µL reaction buffer.

Reactions were stopped by adding 4 µL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 µg/mL proteinase K (Sigma) and incubating for a further 30 min at 37°C. The samples were separated by electrophoresis on 1% agarose gel at room temperature. The gels were stained with ethidium bromide 1 µg/mL in TAE buffer, transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.6. Topoisomerase I-mediated DNA cleavage

Reaction mixtures (20 µL) containing 35 mM Tris-HCl (pH = 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin, 20 ng pBR322 plasmid DNA (Fermentas Life Sciences), 5 U topoisomerase I (human recombinant topoisomerase I, TopoGen) and test compounds were incubated for 60 min at 37 ^{o}C. Reactions were stopped by adding 4 µL of stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 µg/ mL proteinase K (Sigma) and incubating for a further 30 min at 37 ^{o}C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 µg/mL (Sigma) at room temperature in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.7. Topoisomerase II-mediated DNA cleavage

Supercoiled pBR322 plasmid DNA (0.25 µg) was incubated with 10 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 µL reaction buffer.

Reactions were stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.8. Evaluation of apoptotic cell death by Annexin V-FITC and propidium iodide staining

To detect phosphatidylserine translocation from the inner face to the outer surface of plasma membrane, a FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen) was used.

H460 cells (2.0×10^5) were seeded into each cell culture plate in complete growth medium. After incubation for 24 h the test agents were added at the indicated concentrations and cells were incubated for a further 18 h. After treatment, cells were centrifuged and resuspended at 10^6 cells/mL in binding buffer. Cell suspensions (100 μL) were added with Annexin V-FITC and propidium iodide (PI) as indicated by the supplier's instructions, and incubated for 15 min at room temperature in the dark. The populations of Annexin V-negative/PI-negative (viable), Annexin V-positive/PI-negative (early apoptosis), Annexin V-positive/PI-positive (late apoptosis) and Annexin V-negative/PI-positive (necrosis) cells were evaluated by FACSCanto II flow cytometer (Becton–Dickinson, Mountain View, CA).[23]

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The Supplementary Material is available free of charge on the Elsevier Publications Website. Associated content: ^{1}H NMR spectrum, ^{13}C NMR spectrum, ^{1}H-^{13}C HSQC correlation table, ^{1}H-^{13}C HMBC and ^{1}H-^{1}H NOESY correlation table of compound **16**; ^{1}H NMR spectrum detail of compound **22**; HPLC trace of compounds **16** and **23**; Elemental Analyses of compounds **4**-**12**, **15**, **16**, **22** and **23.**

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