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Synthesis and in vitro Evaluation of 3*H*-Pyrrolo[3,2-*f*]-quinolin-9-one Derivatives That Show Potent and Selective Anti-leukemic Activity

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Abstract

A series of new substituted 7-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9-ones were synthesized and evaluated for their antiproliferative activity. The most active derivatives showed high selectivity against human leukemia cell lines and potently inhibited their growth, with GI₅₀ values in the nanomolar range. The active compounds strongly blocked tubulin assembly and colchicine binding to tubulin. Their activities were equal to or greater than that of the reference compound combretastatin A-4. Flow cytometry studies showed that the two most active compounds arrested Jurkat cells in the G₂/M cell-cycle phase in a concentration-dependent manner. This effect was associated with apoptosis, mitochondrial depolarization, generation of reactive oxygen species, activation of caspase-3, and cleavage of the enzyme poly(ADP-ribose) polymerase.

Keywords

anticancer agents; antiproliferative activity; apoptosis; pyrroloquinolinones; tubulin

Introduction

For several years we have studied the pyrroloquinoline nucleus as a promising scaffold for antiproliferative agents,^[1–3] leading to the discovery of the 3*H*-pyrrolo[3,2-*f*]quinoline (PyQ) scaffold (Figure 1) as the most promising framework for the design of potent anticancer compounds. In particular, we found several active agents with *N*-pyrrole alkyl substituents that are derivatives of 7-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9-ones (7-PPyQs).^[3] We showed that 7-PPyQs exhibit strong cytotoxic activity against a wide panel of human tumor cell lines, including multi-drug resistance (MDR)-positive cancer cells. Moreover, in

vivo, the 7-PPyQs exhibit significant inhibition of tumor growth (83 %) in a syngenic hepatocellular carcinoma model in Balb/c mice.[2]

Structure–activity relationship (SAR) studies of various pyrroloquinoline derivatives allowed us to establish some structural features that are crucial for antitumor activity. First, the [3,2-*f*] angular geometry is essential for significant activity, as is the carbonyl group at the 9-position and the side phenyl ring. Moreover, optimal activity requires the presence of an N-alkyl group on the pyrrole ring. Although active compounds were shown to have substituents ranging from ethyl to *n*-pentyl groups as well as a cyclopropylmethyl group, ionizable groups such as dimethylaminoethyl at position N3 or oxygen-containing substituents at positions C2 and C4 caused severe loss of activity. Because previously synthesized compounds[3] had caused cells to arrest in the G₂/M phase of the cell cycle, we suspected that these compounds interact with tubulin, possibly at the colchicine site. Thus, the SAR observations suggested the presence of a deep hydrophobic pocket near the colchicine site.[3] Substituents on the phenyl ring at C7, either electron-withdrawing or -donating groups, had only minor effects on activity.

In an effort to produce additional highly active analogues, we synthesized and evaluated the effects of substitutions at other positions of the 3*H*-pyrrolo[3,2-*f*]quinolin-9-one nucleus. Herein we describe 3*H*-PyQ derivatives with small substituents at the C1 position and with the side phenyl ring shifted from C7 to C8. In addition to synthesizing the simple 8-phenyl-PyQ **33**, substituents were also introduced onto template **33** to examine the effects on the bioactivity of the resulting compounds. We also focused on increasing water solubility relative to previous analogues by inserting suitable substituents at the 1-, 3-, and 7-positions. [3] Such compounds should be valuable for in vivo studies on pharmacokinetics, metabolism, and toxicity.

The newly prepared agents were assayed for their in vitro cytotoxicity against a panel of human solid tumors and leukemic cell lines. The most active compounds were confirmed as inhibitors of tubulin assembly through colchicine site binding, and were found to have major effects on cell-cycle distribution and induction of apoptosis.

Results and Discussion

Chemistry

The new PPyQs were synthesized as shown in Schemes 1–3, generally beginning with an appropriate 5-nitroindole derivative.[1–3] Scheme 1 shows the synthesis of 3,7-disubstituted PPyQs **11–13** by starting from commercial 5-nitroindole, which was first N-alkylated on the pyrrole ring to furnish derivatives **2–4**. [3] The 5-nitroindole was alkylated by ethyl acrylate, trime-thoxybenzyl bromide or bromoethane in the presence of sodium hydride in *N,N*-dimethylformamide (DMF). Intermediates **2–4** were catalytically reduced (Pd/C 10 %, H₂, ethanol) to amines **5–7**. [3] These, in turn, were condensed with ethyl benzoylacetate or ethyl 3-oxo-3-(pyridin-3-yl)propanoate (**1**). We prepared the latter by modifying a published procedure[4] that furnished the β-ketoester **1** in good yield. This compound is present in solution as an equilibrium mixture of the diketo **1 a** and enolic **1 b** forms, as shown by the ¹H NMR spectrum ([D₆]DMSO). This showed two singlets at δ 6.08 (CH) and 12.52 (OH), consistent with an enolic form of the compound. The acrylate derivatives **8–10** were subjected to thermal cyclization (diphenyl ether, 250 °C) to yield compounds **11–13**.

Scheme 2 illustrates the synthesis of 1,3-disubstitued 7-PPyQs **17**, **21**, and **22**. Starting from the known 5-nitroindole **4**, [3] carbaldehyde **14** was prepared by holding at reflux in DMF and phosphoryl chloride. Compound **14** was catalytically reduced (Pd/C 10 %) to 1-methyl-5-aminoindole **15**, which then underwent condensation to yield **16**. The final

cyclization reaction, as in Scheme 1, yielded 1-methyl-3-ethyl-7-PPyQ **17**. Carbaldehyde **14** was also transformed into the corresponding 1-carbonitrile derivative **18** in a one-pot reaction with hydroxyl-amine hydrochloride, pyridine, selenium dioxide, and magnesium sulfate,[5] followed by catalytic reduction to yield amine **19**. Compound **19** was condensed with ethyl benzoylacetate to yield compound **20**, which was thermally cyclized to 1-cyano-3-ethyl-7-PPyQ (**21**). The latter was transformed into 1-carboxy-amide-7-PPyQ **22** by treatment with a 1:1:1 mixture of acetic acid/sulfuric acid/water.

The synthetic route to 8-PPyQs **33–37** is similar to that previously reported for the synthesis of 3-phenylquinolinones (Scheme 3).[6] Commercial ethyl phenylacetates ($R^2 = H$, *m*-methoxy, or *p*-bromo) were formylated with ethyl formate in the presence of sodium hydride[7] to afford intermediates **23**[6]–**25**. These were condensed with 5-aminoindoles **7**, [2] **26**,[3] and **27**[3] in ethanol at room temperature to yield enamine compounds **28–32**. The crude products consisted of a mixture of the *Z* and *E* isomers, as observed by thin-layer chromatography and 1H NMR analysis. These compounds were not separated but underwent thermal cyclization (diphenyl ether, 250 °C) to 8-PPyQs **33–37**.

Biological evaluation

In vitro antiproliferative activities—These new compounds were evaluated for their in vitro anti-proliferative activity against a panel of human tumor cell lines, including both solid and leukemic lines. The previously described[3] 3-ethyl-7-PPyQ (**38**) was used as a reference compound (Figure 2). After continuous drug exposure for 72 h, the compound concentration required for 50 % growth inhibition (GI_{50}) was determined by the MTT colorimetric assay (Table 1). Of the compounds listed, the reference compound **38** showed nanomolar and sub-nanomolar GI_{50} values in all cell lines tested, whereas its regioisomer 3-ethyl-8-PPyQ **34** was inactive ($GI_{50} > 50 \mu M$), as were the other 8-PPyQ derivatives **33** and **35–37**. Clearly, either removal of the side phenyl group from position 7 or shifting it to the 8-position of the tricyclic nucleus is deleterious for activity. Three of the new 7-PPyQ compounds, **11–13**, are active in all cell lines, while an additional three (**17**, **21**, and **22**) have moderate activity only in the leukemic lines. In fact, compound **38** showed GI_{50} values 10–100-fold lower for leukemia cells (GI_{50} : 0.5–2 nM) than for the solid tumor cells (GI_{50} : 0.01–0.06 μM). This difference is even more dramatic for the two most active new compounds, **11** and **12**, for which the GI_{50} values toward the leukemia cells are 0.5–10 nM and toward the solid tumor cells, 0.4–1.4 μM . Both of these compounds have more hydrophilic N-alkyl side chains than other 7-PPyQs.

Of the three compounds bearing a substituent at the 1-position, **21** and **22**, with cyano and carboxyamide groups, respectively, are completely inactive ($GI_{50} > 50 \mu M$) in the solid tumor cells, but show activity at sub-micromolar concentrations in the leukemia lines. Compound **17**, with a methyl group, has only modest activity in the leukemia and HeLa cells. We conclude that a substituent at the 1-position does not abolish antiproliferative activity, as do substituents at position 2.[2] This finding indicates that suitable substituents at the 1-position of 7-PPyQs might result in compounds with high selectivity for leukemic diseases.

Finally, compound **13**, with a pyridine ring in place of the side phenyl group and an ethyl group at the N3 position, like **38**, shows slightly less selectivity toward the leukemic lines than compounds **38**, **11**, **12**, **17**, **21**, and **22**.

Inhibition of tubulin polymerization and colchicine binding—In a previous study, [3] we found that compound **38** and a number of other 7-PpyQs cause cells to arrest at the G_2/M phase of the cell cycle. This finding suggests that the target of these agents is likely to be tubulin, as this is a universal observation in cells treated with anti-tubulin agents. To

determine whether this is true, the most active compounds from the current series, **11–13** and **22**, together with an active agent from the earlier studies (compound **38**), were evaluated for their *in vitro* inhibition of tubulin polymerization and for their inhibitory effect on the binding of [³H]colchicine to tubulin (Table 2).[8,9] For comparison, combretastatin A-4 (CA-4) was examined in simultaneous experiments.

In the assembly assay, compound **11** was found to be the most active ($IC_{50} = 0.75 \mu M$), and it is nearly twice as potent as CA-4 ($IC_{50} = 1.2 \mu M$). Compound **38** is also more inhibitory than CA-4, whereas **12** and **13** are less inhibitory than CA-4. Compound **22** showed only minimal inhibitory activity, with less than 50 % inhibition of tubulin assembly at the highest concentration (40 μM) examined. These effects on tubulin assembly are in good agreement with the relative antiproliferative activities of the compounds.

In the colchicine binding studies, compounds **11** and **38** were roughly equal in activity, but are less potent than CA-4 despite their greater potency as inhibitors of assembly. Such differences are commonly observed, and CA-4 is a particularly potent inhibitor of colchicine binding.[9] Compounds **12** and **13** are much less active as inhibitors of colchicine binding than are CA-4, **11**, and **38**, with convincing inhibition only observed when the inhibitor concentration was increased to 50 μM , 10-fold higher than the concentration of [³H]colchicine in the reaction mixtures.

Cell-cycle analysis—The effects of various concentrations of compounds **11**, **12**, **13**, and **22** on cell-cycle progression were examined with Jurkat leukemic T-lymphocytes (Figure 3). Untreated Jurkat cells showed a classical pattern of proliferation, with large numbers of cells in the G₁ (47.2 %) and S (43.4 %) phases, and fewer cells in the G₂/M (9.4 %) phase. As in our earlier study,[3] the two most active compounds, **11** and **12**, showed a clear G₂/M arrest pattern in a concentration-dependent manner, with a concomitant decrease of cells in the other phases of the cell cycle after 24 h treatment. In particular, as shown in Figure 3, the G₂/M cell population increased from 9.4 % in the control to 77 % with compound **11** at 0.06 μM . The G₁ cells decreased from 47 % in the control to 3 %, while the S-phase cells decreased from 43 to 20 %. Similar behavior was observed for compound **12**, but a similar arrest (65 %) in G₂/M phase required 0.25 μM compound **12**. The other two compounds, **13** and **22**, only induced G₂/M arrest at yet higher concentrations, in good agreement with their lower potency in the tubulin polymerization assay and their lower antiproliferative activity. Further experiments showed that the accumulation of mitotic cells occurs in a time-dependent manner associated with the appearance of a hypodiploid peak (sub-G₁) indicative of apoptosis (data not shown). This observation prompted us to investigate the possible activation of apoptotic mechanisms in the presence of the most active derivatives.

Loss of plasma membrane asymmetry during apoptosis—To better characterize drug-induced apoptosis, we performed a bi-parametric cytofluorimetric analysis using propidium iodide (PI) and annexin V-FITC, which stain DNA and phosphatidylserine (PS) residues, respectively.[10,11] Annexin V is a Ca²⁺-dependent phospholipid binding protein with high affinity for PS. Annexin V staining precedes the loss of membrane integrity that accompanies the final stages of cell death resulting from either apoptotic or necrotic processes. Because the externalization of PS occurs in the earlier stages of apoptosis, annexin V staining identifies apoptosis at an earlier stage than the appearance of sub-G₁ cells. Such cells represent a later stage of cell death involving nuclear changes such as DNA fragmentation.

After drug treatment at various concentrations and time intervals, Jurkat cells were labeled with the two dyes, and the resulting red (PI) and green (FITC) fluorescence was monitored by flow cytometry. Compounds **11** and **12** provoked a remarkable induction of apoptotic

cells after 24 h treatment, whereas the less active compounds **13** and **22** showed lower efficacy (Figure 4). The percentage of annexin-V-positive cells increased further at 48 h. These findings prompted us to further investigate the apoptotic machinery after treatment with compounds **11** and **12**.

Apoptosis is mediated by mitochondrial depolarization. Mitochondria play an essential role in the propagation of apoptosis.[12,13] It is well established that, at an early stage, apoptotic stimuli alter the mitochondrial transmembrane potential ($\Delta\Psi_{mt}$). $\Delta\Psi_{mt}$ was monitored by the fluorescence of the dye JC-1.[14] In the presence of normal cells (high $\Delta\Psi_{mt}$), JC-1 forms fluorescent red (λ 590 nm) aggregates locally and spontaneously that are associated with a large shift in the emission; upon mitochondrial membrane depolarization (low $\Delta\Psi_{mt}$), JC-1 forms monomers that emit at λ 530 nm. Treated Jurkat cells in the presence of derivatives **11** and **12** (0.5 and 0.1 μ M) exhibited a significant increase in the percentage of cells with low $\Delta\Psi_{mt}$ in a concentration- and time-dependent manner relative to the control cells, indicating depolarization of the mitochondrial membrane potential (Figure 5A). The disruption of $\Delta\Psi_{mt}$ is associated with the appearance of annexin V positivity in the treated cells when they are in early apoptotic stage. In fact, the dissipation of $\Delta\Psi_{mt}$ is characteristic of apoptosis and has been observed with both microtubule destabilizing and stabilizing agents in various cell types.[15]

Mitochondrial generation of ROS—Mitochondrial membrane depolarization is associated with mitochondrial production of reactive oxygen species (ROS).[16] Therefore, we investigated whether ROS production increased after treatment with compounds **11** and **12**. We used the fluorescence indicator hydroethidine (HE), the fluorescence of which appears if ROS are generated.[17,18] HE is oxidized by the superoxide anion into the ethidium ion, which emits red fluorescence. Superoxide is produced by mitochondria due to a shift from the normal four-electron reduction of O_2 to a one-electron reduction when cytochrome *c* is released from mitochondria. ROS generation was also measured with the dye 2,7-dichlorodihydrofluorescein diacetate (H_2 -DCFDA), which is oxidized to the fluorescent compound dichlorofluorescein (DCF) by a variety of peroxides including hydrogen peroxide.[18] As shown in Figure 5B,C, compounds **11** and **12**, as expected, induced the production of ROS relative to control cells, in agreement with the dissipation of $\Delta\Psi_{mt}$.

Caspase-3 activation, PARP cleavage, and Bcl-2 down-regulation—Caspases are the central executioners of apoptosis mediated by various inducers.[19,20] Caspases are synthesized as proenzymes, which are activated by cleavage. Caspases-2, -8, -9, and -10 are termed apical and are usually the first to be stimulated in the apoptotic process. These, in turn, activate effector caspases, such as caspase-3 in particular.[21] Exposure of Jurkat cells to compounds **11** or **12** was found to activate caspase-3, as shown in Figure 6, in a time- and concentration-dependent manner.

Poly(ADP-ribose) polymerase (PARP) is a 116 kDa nuclear protein that appears to be involved in apoptosis.[22] This protein is one of the main cleavage targets of caspase-3 both in vitro and in vivo.[22] As illustrated in Figure 7A, immunoblot analysis shows that the typical 89 kDa fragment of PARP increased in treated cells with both compounds in a time-dependent manner. Bcl-2 is a protein extensively investigated as a modulating agent of apoptosis, and it plays a major role in inhibiting apoptosis. Bcl-2 regulates the mitochondrial membrane potential and avoids the subsequent release of cytochrome *c* to prevent caspase activation.[23,24] Therefore, we examined whether the induced apoptosis by **11** and **12** is associated with changes in Bcl-2 expression. As depicted in Figure 7B, flow cytometric analysis shows that both compounds decrease Bcl-2 expression. Altogether, these data

indicate that the induction of apoptosis by these new derivatives is mediated through caspase-3 activation, PARP cleavage, and Bcl-2 down-regulation.

Conclusions

Several 7- and 8-phenylpyrroloquinolinone derivatives were prepared and assayed for their antiproliferative activity on a panel of solid tumor and leukemic cell lines. Depending on the position and nature of substitutions, new SARs on PPyQs were demonstrated that add to those already known. Among these, the most surprising was the inactivity of the regioisomer 8-PPyQs **33–37** toward all cell lines. This indicates that the interaction of the 2-phenylquinolin-4-one moiety in the colchicine site of tubulin either requires the C7 phenyl group or is abolished with a bulky C8 substituent.

Another important SAR is the finding that small hydrophilic groups such as cyano and carboxamide at position 1 of the 3-ethyl-7-PPyQ **38** (compounds **21** and **22**, respectively) retain good antiproliferative activity, but only toward leukemic cells. This should permit the future design of compounds specifically directed against leukemias. Finally, introduction of a less hydrophobic side chain at the N3 or C7 positions of the PyQ nucleus provided compounds **11–13**, which maintain high activity and an antiproliferative profile similar to the reference compound **38**, all of which have greater cytotoxicity against leukemia cells than toward solid tumor cells.

In particular, compound **11** exhibits potent inhibition of tubulin polymerization and is one of the most potent inhibitors of colchicine binding ($IC_{50} = 0.76 \mu M$ for assembly, 44 % inhibition of [3H]colchicine binding when equimolar with tubulin and one fifth the concentration of colchicine in the reaction mixture). Flow cytometry demonstrated that **11** has cellular effects typical for microtubule-interacting agents, causing accumulation of cells in the G₂/M phase of the cell cycle and increased numbers of apoptotic sub-G₁ cells. Moreover, compound **11** is a potent inducer of multiple additional markers of apoptosis in the Jurkat cell line. Apoptosis induced by multiple antimitotic agents has been associated with alteration in a variety of cellular signaling pathways, and compound **11** had similar effects. This agent induced Bcl-2 down-regulation after 24 h treatment. Because Bcl-2 prevents the initiation of the cellular apoptotic program by stabilizing mitochondrial permeability, its loss has multiple consequences. These include loss of $\Delta \Psi_{mt}$, which, in turn, results in an uncoupling of the respiratory chain and mitochondrial efflux of caspase-9 and the apoptosis-inducing factor (AIF). Thus, the proteolytic activation of caspase-3 occurred with subsequent PARP cleavage. Therefore, compound **11**, with its apparent selectivity toward leukemic cell lines, merits further investigation as a potential chemotherapeutic agent.

Experimental Section

Chemistry

Melting points were determined on a Gallenkamp MFB 595 010 M/B capillary melting point apparatus and are not corrected. IR spectra were recorded on a PerkinElmer 1760 FTIR spectrometer using KBr pressed disks; all values are expressed in cm^{-1} . UV/Vis spectra were recorded on a PerkinElmer Lambda UV/Vis spectrometer. 1H NMR spectra were recorded on Bruker spectrometers (300 MHz) with the indicated solvents; chemical shifts (δ) are reported in ppm downfield from $(CH_3)_4Si$ as internal reference. Coupling constants (J) are given in Hz. In the case of multiplets, δ values were measured by starting from the approximate center. Integrals were satisfactorily in line with those expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova (Italy), using a PerkinElmer

elemental analyzer model 240B; results fell in the range of calculated values ± 0.4 %. The analytical data are presented in detail for each final compound. Mass spectra were obtained with a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and an Applied Biosystems Mariner System 5220 LC–MS (nozzle potential: 250.00). Column flash chromatography was performed on Merck silica gel (250–400 mesh ASTM); reactions were monitored by analytical thin-layer chromatography (TLC) using Merck silica gel 60 F₂₅₄ glass plates. Solutions were concentrated in a rotary evaporator under reduced pressure. Starting materials were purchased from Aldrich Chimica and Acros, and solvents from Carlo Erba, Fluka, and Lab-Scan. DMSO was obtained anhydrous by distillation under vacuum and stored on molecular sieves.

Ethyl 3-oxo-3-(pyridin-3-yl)propanoate (1).^[4]—Reagent **1** was obtained by reacting commercial ethyl nicotinate (2.71 mL, 19.8 mmol) with EtOAc (1.5 equiv) in the presence of *t*BuOK (2 equiv) in excess benzene at 80 °C for 6 h. At the end of the reaction, solvent was removed by evaporation, a mixture of ice and H₂O was added, and the mixture was extracted with Et₂O after neutralization with CH₃COOH. The extract was washed with H₂O, dried on anhydrous Na₂SO₄ and evaporated under vacuum. A light-yellow oil product was obtained that was purified by distillation. Yield 45 %; *R*_f = 0.52 (EtOAc/*n*-hexane 8:2); bp: 121–123 °C (0.4 mm); **1a**: ¹H NMR ([D₆]DMSO): δ = 1.17 (t, 3 H, *J* = 7.1 Hz, CH₃), 4.12 (q, 2 H, *J* = 7.1 Hz, CH₂), 4.27 (s, 2 H, CH₂), 7.58 (ddd, 1 H, *J* = 0.8, 4.8, and 8.0 Hz, 5-H), 8.29 (dt, 1H, *J* = 2.3 Hz, 8.01, 4-H), 8.76 (dd, 1 H, *J* = 4.7 and 6.5 Hz, 6-H), 9.12 (dd, 1H, *J* = 0.6 and 2.3 Hz, 2-H); **1b**: 6.08 (s, 1H, CH), 12.52 (s, 1H, OH).

Ethyl 3-(5-nitro-1*H*-indol-1-yl)propanoate (2)—An amount corresponding to 35 mmol NaH (60% dispersion in mineral oil) was placed in a 100 mL round-bottomed flask and washed with toluene (3 × 10 mL). A solution of commercial 5-nitroindole (12 mmol) in dry toluene (60 mL) was dropped into the flask, and the initial yellow color changed to red, with the formation of H₂ gas. After 30 min at room temperature, a solution of ethyl acrylate (12 mmol) in dry toluene (10 mL) was added, and the reaction mixture was held at reflux for 4 h. Next, the reaction mixture was cooled to 0 °C, a few drops of absolute EtOH were added, the volatiles were removed, and the residue was partitioned between H₂O (15 mL) and CHCl₃ (60 mL). The organic extract was evaporated under vacuum. Yellow–orange solid, yield 76 %; mp: 65–66 °C; *R*_f = 0.71 (eluent benzene/*n*-hexane/EtOAc 1:1:1); ¹H NMR ([D₆]DMSO): δ = 1.08 (t, 3 H, *J* = 7.1 Hz, CH₃), 2.88 (t, 2H, *J* = 6.7 Hz, CH₂), 3.99 (q, 2 H, *J* = 7.1 Hz, CH₂), 4.56 (t, 2H, *J* = 6.7 Hz, CH₂), 6.80 (d, 1 H, *J*_{2,3} = 3.2 Hz, 2-H), 7.81 (m, 2H, 7-H, 3-H), 8.02 (dd, 1H, *J*_{4,6} = 2.2 Hz, *J*_{6,7} = 9.1 Hz, 6-H), 8.58 (d, 1H, *J*_{4,6} = 2.2 Hz, 4-H); HRMS (ESI) calcd for C₁₃H₁₅N₂O₄ [*M*+H]⁺ *m/z* 263.0987, found 263.1012.

1-(3,4,5-Trimethoxybenzyl)-5-nitro-1*H*-indole (3)—An amount corresponding to 37 mmol NaH (60 % dispersion in mineral oil) was placed in a three-necked 100 mL round-bottomed flask and washed with toluene (3 × 10 mL). A solution of commercial 5-nitroindole (12.3 mmol) in 20–30 mL dry DMF was dropped into the flask, and the initial yellow color changed to red, with the formation of H₂ gas. After 30 min at room temperature, a solution of trimethoxybenzyl chloride (25 mmol) in dry DMF was added, and the reaction mixture was left stirring for 20–48 h with monitoring of the ongoing reaction by TLC analysis (eluent: toluene/*n*-hexane/EtOAc 1:1:0.3). At the end, the solvent was evaporated, and the residue was extracted with EtOAc. The organic phase, washed with H₂O and dried over anhydrous Na₂SO₄, was dried under vacuum. Yield 98 %; *R*_f = 0.50 (benzene/*n*-hexane/EtOAc 1:1:1); mp: 158–160 °C; ¹H NMR ([D₆]DMSO): δ = 3.59 (s, 3H, 4'-OCH₃), 3.69 (s, 6 H, 3'- and 5'-OCH₃), 5.41 (s, 2 H, CH₂), 6.65 (s, 2 H, 2'- and 6'-H), 6.80 (d, 1 H, *J*_{2,3} = 3.2 Hz, 2-H), 7.81 (m, 2 H, 7-H and 3-H), 8.02 (dd, 1H, *J*_{4,6} = 2.2 Hz, *J*_{6,7} = 9.1, 6-H),

8.58 (d, 1H, $J_{4,6} = 2.2$ Hz, 4-H); HRMS (ESI) calcd for $C_{18}H_{19}N_2O_5$ $[M+H]^+$ m/z 343.1249, found 343.1364.

General procedure for the synthesis of 5-aminoindole derivatives 5–7[3]—A

solution of a nitroindole **2–4**[3] (5.87 mmol) in 400 mL of EtOH was dropped into a suspension of 10 % Pd/C (125 mg) saturated with H_2 in 200 mL EtOH. The mixture was stirred at room temperature with hydrogen at atmospheric pressure for 3 h, and the reaction was monitored by TLC analysis (EtOAc/*n*-hexane 1:3). The catalyst was then removed by filtration, and the filtrate was evaporated under reduced pressure to dryness to give the corresponding aminoindole. The nearly pure residue (90% yield) was used in the next reaction without purification.

Ethyl 3-(5-amino-1*H*-indol-1-yl)propanoate (5)—Brown dense liquid; yield 98 %; $R_f = 0.48$ (benzene/*n*-hexane/EtOAc 1:1:1); 1H NMR ($[D_6]$ DMSO): $\delta = 1.12$ (t, 3H, $J = 7.1$ Hz, CH_3), 2.75 (t, 2H, $J = 6.7$ Hz, CH_2), 4.01 (q, 2H, $J = 7.1$ Hz, CH_2), 4.63 (t, 2H, $J = 6.7$ Hz, CH_2), 5.18 (bs, 2H, NH_2), 6.15 (dd, 1H, $J_{3,2} = 3.1$ Hz, $J_{3,4} = 0.8$ Hz, 3-H), 6.52 (dd, 1H, $J_{6,7} = 8.6$ Hz, $J_{6,4} = 2.1$ Hz, 6-H), 6.67 (dd, 1H, $J_{4,6} = 2.1$ Hz, $J_{4,3} = 0.6$ Hz, 4-H), 7.16 (d, 1H, $J_{7,6} = 8.6$ Hz, 7-H), 7.19 (d, 1H, $J_{2,3} = 3.0$ Hz, 2-H).

1-(3,4,5-Trimethoxybenzyl)-5-amino-1*H*-indole (6)—Reddish waxy solid; yield 90 %; $R_f = 0.58$ (EtOAc/*n*-hexane 7:3); 1H NMR ($[D_6]$ DMSO): $\delta = 3.59$ (s, 3H, 4'-OCH₃), 3.69 (s, 6H, 3'- and 5'-OCH₃), 4.40 (bs, 2H, NH_2), 5.18 (s, 2H, CH_2), 6.53 (s, 2H, 2'- and 6'-H), 6.64 (m, 3- and 7-H), 6.78 (d, 1H, $J_{4,6} = 2.1$ Hz, 4-H), 7.22 (dd, 1H, $J_{6,7} = 8.6$ Hz, 6-H), 7.32 (d, 1H, $J_{2,3} = 3.2$ Hz, 2-H).

General procedure for the synthesis of ethyl 3-(1-substituted-indoleamino)-3-phenylacrylates (8–10)—In a 50 mL round-bottomed flask, 3–4 mmol 3-substituted

aminoindole **5–7**[2] in 10–20 mL absolute EtOH were condensed with an equimolar quantity of commercial ethyl benzoylacetate for **8** and **9** or ethyl 3-oxo-3-(pyridin-3-yl)propanoate for **10** with 1 mL glacial CH_3COOH and 100 mg Drierite. The mixture was held at reflux for ~24 h, the reaction being monitored by TLC analysis (CH_2Cl_2 /EtOAc 8:2). As the reaction did not come to completion, after 24 h the mixture was cooled and filtered to remove the Drierite. The resulting solution was evaporated to dryness under vacuum, and the residue was purified by flash chromatography.

Ethyl 3-[1-(3-ethoxy-3-oxopropyl)-1*H*-indol-5-ylamino]-3-phenyl-acrylate (8)—

Brown dense liquid; yield 68 %; $R_f = 0.83$ (CH_2Cl_2 /EtOAc 8:2); 1H NMR ($[D_6]$ DMSO): $\delta = 1.11$ (t, 3H, $J = 7.1$ Hz, CH_3), 1.16 (t, 3H, $J = 7.1$ Hz, CH_3), 2.80 (t, 2H, $J = 6.7$ Hz, CH_2), 4.01 (q, 2H, $J = 7.1$ Hz, CH_2), 4.13 (q, 2H, $J = 7.1$ Hz, CH_2), 4.38 (t, 2H, $J = 6.7$ Hz, CH_2), 4.83 (s, 1H, CH), 6.20 (d, 1H, $J_{3,2} = 3.1$ Hz, 3-H), 6.60 (dd, 1H, $J_{6,7} = 8.8$ Hz, $J_{6,4} = 2.1$ Hz, 6-H), 6.97 (d, 1H, $J_{4,6} = 2.1$ Hz, 4-H), 7.30 (m, 7H, ar), 10.25 (s, 1H, NH); HRMS (ESI) calcd for $C_{24}H_{27}N_2O_4$ $[M+H]^+$ m/z 407.1926, found 407.1997.

Ethyl 3-phenyl-3-[1-(3,4,5-trimethoxybenzyl)-1*H*-indol-5-ylamino]acrylate (9)—

Dark-green liquid; yield 70 %; $R_f = 0.96$ (CH_2Cl_2 /EtOAc 8:2); 1H NMR ($[D_6]$ DMSO): $\delta = 1.23$ (t, 3H, $J = 7.1$ Hz, CH_3), 3.59 (s, 3H, 4'-OCH₃), 3.69 (s, 6H, 3'- and 5'-OCH₃), 4.12 (q, 2H, $J = 7.1$ Hz, CH_2), 4.83 (s, 1H, CH), 5.18 (s, 2H, CH_2), 6.20 (d, 1H, $J_{3,2} = 3.1$ Hz, 3-H), 6.60 (dd, 1H, $J_{6,7} = 8.8$ Hz, $J_{6,4} = 2.1$ Hz, 6-H), 6.53 (s, 2H, 2'- and 6'-H), 6.97 (d, 1H, $J_{4,6} = 2.1$ Hz, 4-H), 7.30 (m, 7H, ar), 10.20 (s, 1H, NH); HRMS (ESI) calcd for $C_{29}H_{31}N_2O_5$ $[M+H]^+$ m/z 487.2188, found 487.2410.

Ethyl 3-(1-ethyl-1*H*-indol-5-ylamino)-3-(pyridin-3-yl)acrylate (10)—Brown dense liquid; yield 40 %; R_f = 0.75 (EtOAc/*n*-hexane 8:2); ^1H NMR ($[\text{D}_6]\text{DMSO}$): δ = 1.23 (t, 3H, J = 7.3 Hz, CH_3), 1.29 (t, 3 H, CH_3), 4.11 (m, 4H, J = 7.1 Hz, $2 \times \text{CH}_2$), 4.90 (s, 1H, CH), 6.22 (dd, 1 H, $J_{3,2}$ = 3.1 and $J_{3,4}$ = 0.6 Hz, 3-H), 6.62 (dd, 1H, $J_{6,7}$ = 8.8 and $J_{6,4}$ = 2.01 Hz, 6-H), 6.97 (d, 1 H, $J_{4,6}$ = 2.0 Hz, 4-H), 7.31 (m, 4H, ar), 7.40 (d, 1 H, $J_{3,2}$ = 3.1 Hz, 2-H), 7.46 (d, 1 H, $J_{6,7}$ = 8.8 Hz, 7-H), 10.35 (s, 1 H, NH); HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{22}\text{N}_3\text{O}_2$ $[M+\text{H}]^+$ m/z 336.1667, found 336.1749.

General procedure for the synthesis of 3-substituted 7-phenyl-and 7-pyridin-3*H*-pyrrolo[3,2-*f*]quinolin-9-(6*H*)ones 11–13—In a two-necked round-bottomed flask, 50 mL Ph_2O was heated at boiling point; 1–2 mmol of acrylates **8–10** were then added portion-wise, and the mixture was held at reflux for 30 min. After cooling to 60 °C, the separated precipitate was collected by filtration and washed several times with Et_2O . In all cases, the collected products were purified by flash chromatography (EtOAc/MeOH 8:2) or re-crystallized from a suitable solvent.

Ethyl 3-(9-oxo-7-phenyl-6*H*-pyrrolo[3,2-*f*]quinolin-3(9*H*)-yl)propanoate (11)—Yellow solid; yield 45 %; mp: 202–204 °C; R_f = 0.60 (EtOAc/MeOH 9:1); mp: 249–250 °C (dec.); ^1H NMR (CD_3OD): δ = 1.17 (t, 3 H, CH_3), 2.91 (d, 2 H, CH_2), 4.09 (q, 2 H, CH_2), 4.64 (d, 2 H, CH_2), 6.69 (s, 1 H, 8-H), 7.46 (d, 1H, $J_{1,2}$ = 3.1 Hz, 1-H), 7.59 (m, 4 H, ar), 7.67 (d, 1 H, $J_{1,2}$ = 3.1 Hz, 2-H) 7.82 (m, 2 H, 2'- and 6'-H), 7.93 (d, 1 H, $J_{4,5}$ = 8.9 Hz, 4-H); ^{13}C NMR (CD_3OD): δ = 14.8, 36.7, 43.5, 62.3, 105.9, 109.8, 113.6, 118.3, 126.1, 128.1, 130.4, 131.8, 136.4, 173.3, 180.2; HRMS (ESI) calcd for $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}_3$ $[M+\text{H}]^+$ m/z 361.1547, found 361.1532; anal. calcd for $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}_3$: C 73.32, H 5.59, N 7.77, found: C 73.56, H 5.42, N 7.43.

7-Phenyl-3-(3,4,5-trimethoxybenzyl)-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (12)—Light-brown solid; yield 40 %; mp: 249–250 °C; R_f = 0.55 (eluent EtOAc/MeOH 9:1); ^1H NMR (CD_3OD): δ = 3.91 (s, 9 H, $3 \times \text{OCH}_3$), 5.70 (s, 2 H, CH_2), 6.60 (s, 2 H, 2'- and 6'-H), 6.90 (s, 1 H, 8-H), 7.77 (m, 5 H, ar), 7.92 (d, 1H, $J_{1,2}$ = 3.1 Hz, 2-H), 8.03 (m, 3 H, 4-H, 2''- and 6''-H); ^{13}C NMR (CD_3OD): δ = 51.2, 56.5, 61.1, 105.1, 128.6, 130.7, 135.9, 154.8, 180.3; HRMS (ESI) calcd for $\text{C}_{27}\text{H}_{24}\text{N}_2\text{O}_4$ $[M+\text{H}]^+$ m/z 441.1809, found 441.1668; anal. calcd for $\text{C}_{27}\text{H}_{24}\text{N}_2\text{O}_4$: C 73.62, H 5.49, N 6.36, found: C 73.81, H 5.39, N 6.15.

3-Ethyl-7-(pyridin-3-yl)-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (13)—Beige solid; yield 35 %; mp: 278–280 °C (dec.) (MeOH); R_f = 0.46 (eluent EtOAc/MeOH 8:2); ^1H NMR (CD_3OD): δ = 1.38 (t, 3 H, CH_3), 4.25 (q, 2 H, CH_2), 6.56 (bs, 1 H, 8-H), 7.35 (d, 1H, $J_{1,2}$ = 2.9 Hz, 1-H), 7.44 (d, 1 H, $J_{4,5}$ = 9.0 Hz, 5-H), 7.53 (m, 2H, 2- and 5'-H), 7.79 (d, 1 H, $J_{4,5}$ = 9.0 Hz, 4-H), 8.14 (m, 1 H, 6'-H), 8.60 (dd, 1H, J = 1.5 and 4.9 Hz, 4'-H), 8.88 (d, 1H, J = 2.3 Hz, 2'-H); ^{13}C NMR (CD_3OD): δ = 16.5, 42.2, 105.7, 117.5, 125.5, 129.5, 133.3, 148.7, 151.7, 182.9; HRMS (ESI) calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}$ $[M+\text{H}]^+$ m/z 290.1288, found 290.1385; anal. calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}$: C 74.72, H 5.23, N 14.52, found: C 74.95, H 5.36; 14.11

1-Ethyl-5-nitro-1*H*-indole-3-carbaldehyde (14)—A solution of 1-ethyl-5-nitro-1*H*-indole **4**[3] (1.5634 g, 8.22 mmol) in DMF was dropped into a mixture of 2 mL POCl_3 and 3 mL DMF cooled at 10 °C. The temperature was raised to 35–40 °C with stirring, and the reaction continued until the starting material was no longer detectable by TLC. The volatile products were removed in vacuo, and the residue was diluted with a mixture of H_2O and ice, followed by prior basification with 6 % NaOH. A heavy precipitate formed, which was removed by filtration and washed with a mixture of H_2O and EtOAc. The organic phase of the filtrate was separated and evaporated to give a yellow residue (1.0052 g). The precipitate was resuspended in EtOAc, and the mixture was stirred overnight. It was then filtered, and

the EtOAc solution was dried under vacuum to give another 0.512 g of product. Light-yellow solid; yield 87 %; mp: 178–180 °C; R_f = 0.46 (EtOAc/*n*-hexane 7:3); ^1H NMR ([D₆]DMSO): δ = 1.44 (t, 3H, J = 7.1 Hz, CH₃), 4.40 (q, 2 H, J = 7.1 Hz, CH₂), 7.90 (d, 1 H, $J_{6,7}$ = 9.1 Hz, 7-H), 8.20 (dd, 1 H, $J_{4,6}$ = 2.2 and $J_{6,7}$ = 9.1 Hz, 6-H), 8.65 (s, 1 H, 2-H), 8.95 (d, 1H, $J_{4,6}$ = 2.1 Hz, 4-H), 10.00 (s, 1 H, HCO); HRMS (ESI) calcd for C₁₁H₁₁N₂O₃ [M + H]⁺ m/z 219.0725, found 219.0757.

1-Ethyl-3-methyl-1*H*-indole-5-amine (15)—As with the aminoindole compounds **5–7**, the nitroindole carbaldehyde **14** was catalytically reduced, at 40–50 °C for 10–14 h, to give a dense, red liquid product. Yield 77 %; R_f = 0.74 (EtOAc/*n*-hexane 8:2); ^1H NMR ([D₆]DMSO): δ = 1.05 (t, 3 H, CH₃), 2.11 (s, 3 H, CH₃), 3.99 (q, 2 H, CH₂), 4.35 (bs, 2 H, NH₂), 6.49 (dd, 1 H, $J_{6,7}$ = 8.2 and $J_{6,4}$ = 2.3 Hz, 6-H), 6.60 (d, 1H, $J_{6,4}$ = 2.3, 4-H), 6.91 (s, 1H, 2-H), 7.07 (bd, 1 H, $J_{6,7}$ = 8.2 Hz, 7-H).

Ethyl 3-(1-ethyl-3-methyl-1*H*-indol-5-ylamino)-3-phenylacrylate (16)—As with the acrylate compounds **8–10**, aminoindole **15** was condensed with ethyl benzoylacetate to give a brown liquid product. Yield 56 %; R_f = 0.91 (CH₂Cl₂/EtOAc 8:2); ^1H NMR ([D₆]DMSO): δ = 1.23 (t, 3 H, J = 7.3 Hz, CH₃), 1.29 (t, 3 H, CH₃), 2.03 (s, 3H, CH₃), 4.11 (m, 4 H, J = 7.1 Hz, 2 × CH₂), 4.83 (s, 1 H, CH), 6.54 (dd, 1 H, $J_{6,7}$ = 8.7 and $J_{6,4}$ = 1.8 Hz, 6-H), 6.87 (d, 1 H, $J_{4,6}$ = 1.9 Hz, 4-H), 7.16 (d, 1 H, $J_{7,6}$ = 8.8 Hz, 7-H), 7.31 (m, 6H, ar), 10.25 (s, 1 H, NH); HRMS (ESI) calcd for C₂₂H₂₅N₂O₂ [M + H]⁺ m/z 349.1871, found 349.2023.

3-Ethyl-1-methyl-7-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (17)—As with the pyrroloquinolinone derivatives **11–13**, the acrylate **16** was cyclized to give a light-brown solid product. Yield 30 %; R_f = 0.77 (EtOAc/MeOH 8:2); mp: 163–165 °C; ^1H NMR (CD₃OD): δ = 1.58 (t, 3 H, J = 7.3 Hz, CH₃), 2.92 (s, 3H, CH₃), 4.31 (q, 2H, J = 7.3 Hz, CH₂), 6.73 (s, 1H, 2-H), 7.33 (bs, 1 H, 8-H), 7.56 (d, 1H, J = 8.9 Hz, 5-H), 7.64 (m, 3 H, 3', 4'- and 5'-H), 7.85 (m, 3 H, 4-, 2'- and 6'-H); ^{13}C NMR (CD₃OD): δ = 12.3, 16.2, 42.3, 105.6, 125.6, 129.5, 148.8, 180.0; HRMS (ESI) calcd for C₂₀H₁₈N₂O [M + H]⁺ m/z 303.1492, found 303.1722; anal. calcd for C₂₀H₁₈N₂O: C 79.44, H 6.00, N 9.26, found: C 79.61, H 6.23, N 9.01.

1-Ethyl-5-nitro-1*H*-indole-3-carbonitrile (18)—In a round-bottomed flask, 5-nitroindolo-3-carbaldehyde **14** (1.539 g, 7.05 mmol) was dissolved in 5 mL anhydrous DMF. To this was added 0.514 g (7.4 mmol) NH₂OH·HCl and 0.57 mL (7.4 mmol) anhydrous pyridine. The reaction mixture was stirred at 80 °C under N₂ for ~ 2 h, until the starting material disappeared and another spot with higher R_f formed (TLC, EtOAc/*n*-hexane 7:3). At this point, 0.821 g of SeO₂ and 0.400 g of MgSO₄ were added to the hot reaction mixture. Stirring continued at 80 °C for another 2.5 h. The reaction mixture was cooled and filtered, and the filtered solution was evaporated to dryness under vacuum. The residue was resuspended in cold H₂O, and the suspension was re-filtered. The collected yellow solid was washed several times with H₂O and finally dried. Yield 60 %; R_f = 0.53 (EtOAc/*n*-hexane 7:3); mp: 158–161 °C; ^1H NMR ([D₆]DMSO): δ = 1.41 (t, 3H, J = 7.1 Hz, CH₃), 4.38 (q, 2 H, J = 7.1 Hz, CH₂), 7.96 (d, 1 H, $J_{6,7}$ = 9.1 Hz, 7-H), 8.20 (dd, 1 H, $J_{4,6}$ = 2.2 and $J_{6,7}$ = 9.1 Hz, 6-H), 8.51 (d, 1H, $J_{4,6}$ = 2.1 and $J_{6,7}$ = 9.1 Hz, 4-H), 8.64 (s, 1 H, 2-H); HRMS (ESI) calcd for C₁₁H₁₀N₃O₂ [M + H]⁺ m/z 216.0728, found 216.0783.

5-Amino-1-ethyl-1*H*-indole-3-carbonitrile (19)—As with the aminoindole compounds **5–7** and **15**, the nitroindole carbonitrile **18** was catalytically reduced at 40–50 °C for 10–24 h to give a yellow semi-solid product. Yield 98 %; R_f = 0.66 (EtOAc/*n*-hexane 8:2); ^1H NMR ([D₆]DMSO): δ = 1.36 (t, 3H, J = 7.2 Hz, CH₃), 4.15 (q, 2 H, J = 7.2 Hz, CH₂), 4.96 (bs, 2 H, NH₂), 6.65 (dd, 1 H, $J_{4,6}$ = 1.9 and $J_{6,7}$ = 8.7 Hz, 6-H), 6.72 (d, 1 H, $J_{6,4}$ = 1.9 Hz,

4-H), 7.35 (d, 1 H, $J_{6,7} = 8.7$ Hz, 7-H), 8.02 (s, 1 H, HC-2); HRMS (ESI) calcd for $C_{11}H_{12}N_3$ $[M+H]^+$ m/z 186.0987, found 186.1047.

Ethyl 3-(3-cyano-1-ethyl-1H-indol-5-ylamino)-3-phenylacrylate (20)—As with the acrylate compounds **8–10** and **16**, aminoindole **19** was condensed with benzoylacetate to give a dense yellow liquid. Yield 52 %; $R_f = 0.84$ (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): $\delta = 1.23$ (t, 3 H, $J = 7.3$ Hz, CH_3), 1.29 (t, 3 H, CH_3), 4.11 (m, 4 H, $J = 7.1$ Hz, $2 \times CH_2$), 4.92 (s, 1H, CH), 6.67 (dd, 1H, $J_{6,7} = 8.7$ and $J_{6,4} = 2.0$ Hz, 6-H), 6.95 (d, 1 H, $J_{4,6} = 1.9$ Hz, 4-H), 7.35 (d, 1 H, $J_{7,6} = 8.7$ Hz, 7-H), 7.31 (m, 6 H, ar), 10.25 (s, 1 H, NH).

3-Ethyl-9-oxo-7-phenyl-6,9-dihydro-3H-pyrrolo[3,2-f]quinoline-1-carbonitrile (21)—As with the pyrroloquinolinone derivatives **8–10** and **17**, the acrylate **20** was cyclized to give a brown solid. Yield 24 %; mp: 209 °C; $R_f = 0.33$ (EtOAc/MeOH 9:1, light-blue fluorescent spot at λ_{365} nm); 1H NMR (CD_3OD): $\delta = 1.63$ (t, 3 H, $J = 7.3$ Hz, CH_3), 4.55 (q, 2H, $J = 7.3$ Hz, CH_2), 6.76 (s, 1H, 2-H), 7.70 (m, 3H, 3', 4'- and 5'-H), 7.84 (d, 1 H, $J_{4,5} = 8.9$ Hz, 4-H), 7.93 (m, 2 H, 2'- and 6'-H), 8.11 (d, 1H, $J_{4,5} = 9.1$ Hz, 5-H), 8.31 (s, 1 H, 2-H); ^{13}C NMR (CD_3OD): $\delta = 15.9$ (CH_3), 43.4 (CH_2), 89.9, 118.8 (CN), 128.8 (C-2', C-3', C-5', C-6'); HRMS (ESI) calcd for $C_{20}H_{15}N_3O_2$ $[M+H]^+$ m/z 314.1288, found 314.1301; anal. calcd for $C_{20}H_{15}N_3O$: C 76.66, H 4.82, N 13.41, found: C 76.94, H 4.53, N 13.11.

3-Ethyl-9-oxo-7-phenyl-6,9-dihydro-3H-pyrrolo[3,2-f]quinoline-1-carboxamide (22)— CH_3COOH , H_2SO_4 , and H_2O (2 mL each) were added in sequence to a 100 mL flask containing 0.188 g cyano compound **21** (0.60 mmol). The mixture was heated at 70 °C for 16 h. At the end (followed by TLC, EtOAc/MeOH 8:2), the reaction was stopped by adding a mixture of H_2O and ice and left at 4 °C for 4 h. The formed precipitate was collected, washed with H_2O , and dried to yield a brown solid. Yield 37 %; mp: 223–225 °C; $R_f = 0.56$ (EtOAc/MeOH 9:1, light-blue fluorescent spot at λ_{365} nm); 1H NMR (CD_3OD): $\delta = 1.53$ (t, 3 H, $J = 7.3$ Hz, CH_3), 4.43 (q, 2 H, $J = 7.3$ Hz, CH_2), 6.91 (s, 1H, 2-H), 7.61 (d, 1H, $J_{4,5} = 9.1$ Hz, 5-H), 7.82 (m, 3H, 3', 4'- and 5'-H), 8.11 (d, 1H, 6-H), 8.22 (s, 1H, 8-H); HRMS (ESI) calcd for $C_{20}H_{17}N_3O_2$ $[M+H]^+$ m/z 332.1541, found 332.1444; anal. calcd for $C_{20}H_{17}N_3O_2$: C 72.49, H 5.17, N 12.68, found: C 72.61, H 4.92, N 12.29.

General procedure for the synthesis of ethyl 3-oxo-2-phenylpropanoate derivatives (23[7]–25)—NaH (60% in oil, 4 equiv in mmol) was added portionwise to a mixture of commercial ethyl phenylacetate, *m*-methoxyphenylacetate or *p*-bromophenylacetate (3.1–6.1 mmol) and ethyl formate (61.4–121.3 mmol). This mixture was stirred at room temperature under N_2 for 24 h (TLC, $CHCl_3/MeOH$ 9.9:0.1), then poured into a mixture of ice and H_2O , acidified with HCl, and extracted with EtOAc. The extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield colored oils.

Ethyl 3-oxo-2-(*m*-methoxyphenyl)propanoate (24)—Orange oil; yield 87 %; $R_f = 0.84$ (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): $\delta = 1.18$ (t, 3H, $J = 7.1$ Hz, CH_3), 3.72 (s, 1H, OCH_3), 4.09 (q, 2H, $J = 7.1$ Hz, CH_2), 6.78 (dd, 1H, $J = 2.7$ and 8.1, 4-H), 6.85 (m, 2H, 2- and 6-H), 7.21 (dd, 1H $J = 8.1$, 5-H), 7.84 (s, 1H, CH), 10.95 (bs, 1 H, CHO); HRMS (ESI) calcd for $C_{12}H_{15}O_4$ $[M+H]^+$ m/z 223.0926, found 223.0939.

Ethyl 3-oxo-2-(*p*-bromophenyl)propanoate (25)—Yellow oil; yield 67 %; $R_f = 0.86$ (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): $\delta = 1.18$ (t, 3 H, $J = 7.1$ Hz, CH_3), 4.10 (q, 2 H, $J = 7.1$ Hz, CH_2), 7.26 (d, 1 H, $J = 8.4$ Hz, 5- and 6-H), 7.49 (d, 1H, $J = 8.6$ Hz, 2- and 3-

H), 7.87 (s, 1 H, CH), 11.194 (bs, 1 H, CHO); HRMS (ESI) calcd for $C_{11}H_{11}O_3Br$ $[M+H]^+$ m/z 272.9949, found 270.9987 and 272.9994.

General procedure for the synthesis of 3-(1*H*-indol-5-ylamino-2-phenylpropanoate derivatives (28–32)—A solution of aminoindole (7,[2] 26,[3] or 27[3]) (1.9–2.2 mmol) in 20 mL absolute EtOH was added to aldehyde compounds (23,[7] 24, or 25) in equimolar ratio dissolved in a minimal quantity of EtOH. Drierite (100 mg) was added to the mixture, and the mixture was stirred at room temperature for 10–15 h. At the end (TLC, $CHCl_3/MeOH$ 9:1) the reaction mixture was filtered, and the filtrate was concentrated under vacuum to give semi-solid products.

Ethyl 3-(1*H*-indol-5-ylimino)-2-phenylpropanoate (28)—Dark oil; yield 57 %; R_f = 0.46 and 0.83 (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): δ = 1.28 (t, 3H, J = 7.2 Hz, CH_3), 4.14 (q, 2 H, J = 7.2 Hz, CH_2), 7.44–7.18 (m, 10 H, ar), 7.64 (d, 1H, J = 13.0 Hz, =CH), 8.05 (d, 1H, J = 13.9 Hz, CH), 8.43 (d, 1H, J = 13.6 Hz, CH), 10.34 (d, 1 H, J = 13.0 Hz, =CH), 11.04 (bs, 1 H, NH); HRMS (ESI) calcd for $C_{19}H_{19}N_2O_2$ $[M+H]^+$ m/z 307.1402, found 307.1444.

Ethyl 3-(1-ethyl-1*H*-indol-5-ylimino)-2-phenylpropanoate (29)—Black oil; yield 74 %; R_f = 0.30 and 0.85 (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): δ = 1.20 (m, 6H, J = 7.2 Hz, $2 \times CH_3$), 4.14 (m, 6 H, J = 7.2 Hz, $2 \times CH_2$), 6.34 (t, 2 H, J = 2.5 Hz, 1- and 2-H), 7.27–7.45 (m, 8 H, ar), 7.65 (d, 1 H, J = 12.9 Hz, =CH), 8.05 (d, 1H, J = 13.5 Hz, NH), 8.45 (d, 1 H, J = 13.5 Hz, NH), 10.35 (d, 1 H, J = 12.9 Hz, =CH); HRMS (ESI) calcd for $C_{21}H_{23}N_2O_2$ $[M+H]^+$ m/z 335.1715, found 335.1707.

Ethyl 3-(1-cyclopropylmethyl-1*H*-indol-5-ylimino)-2-phenylpropanoate (30)—Black liquid; yield 95 %; R_f = 0.58 and 0.92 (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): δ = 0.33 (m, 4H, $2 \times CH_2$), 0.47 (m, 4 H, $2 \times CH_2$), 1.30–1.90 (m, 12 H, $2 \times CH$), 4.15–4.41 (m, 6 H, $2 \times CH_2$), 7.14–7.47 (m, 10 H, ar), 8.06 (d, 1H, J = 13.0 Hz, =CH), 8.40 (d, 1 H, J = 13.7 Hz, NH), 8.60 (d, 1H, J = 13.7 Hz, NH), 10.33 (d, 1H, J = 13.0 Hz, =CH).

Ethyl 3-(1-ethyl-1*H*-indol-5-ylimino)-2-(3-methoxyphenyl)propanoate (31)—Black liquid; yield 75 %; mp: 249–250 °C; R_f = 0.64 and 0.85 (EtOAc/*n*-hexane 9:1); 1H NMR ($[D_6]$ DMSO): δ = 1.33 (m, 6 H, $2 \times CH_3$), 4.14 (m, 4 H, $2 \times CH_2$), 6.82–7.45 (m, 9 H, ar), 7.66 (d, 1 H, J = 13.2 Hz, =CH), 8.04 (d, 1 H, J = 13.6 Hz, CH), 8.45 (d, 1 H, J = 13.9 Hz, CH), 10.36 (d, 1 H, J = 13.2 Hz, =CH); HRMS (ESI) calcd for $C_{22}H_{25}N_2O_3$ $[M+H]^+$ m/z 365.1820, found 365.1796.

Ethyl 2-(4-Bromophenyl)-3-(1-ethyl-1*H*-indol-5-ylimino)propanoate (32)—Black oil; yield 73.93 %; R_f = 0.52 and 0.92 (EtOAc/*n*-hexane 8:2); 1H NMR ($[D_6]$ DMSO): δ = 1.30 (m, 6H, $2 \times CH_3$), 4.17 (m, 4 H, $2 \times CH_2$), 7.57–7.07 (m, 9 H, ar), 7.69 (d, 1H, J = 13.2 Hz, =CH), 8.05 (d, 1H, J = 13.6 Hz, NH), 8.65 (d, 1H, J = 13.6 Hz, NH), 10.38 (d, 1H, J = 13.2 Hz, =CH); HRMS (ESI) calcd for $C_{21}H_{22}N_2O_2Br$ $[M+H]^+$ m/z 414.0766, found 413.0823 and 415.0799.

General procedure for the synthesis of 8-phenyl-pyrrolo[3,2-*f*]quinolinones (33–37)—In a two-necked round-bottomed flask, Ph_2O (30 mL) was heated at boiling point; 1–2 mmol of propanoates 28–32 were then added in portions, and the mixture was held at reflux for 15 min. After cooling to 30°C, the light precipitate slowly separated and was then collected by filtration and washed several times with Et_2O . In all cases, the collected products were purified by recrystallization from a suitable solvent.

8-Phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (33)—Light-brown solid; yield 87.98 %; mp: 178–190 °C (MeOH); R_f = 0.28 (EtOAc/*n*-hexane 8:1, violet fluorescent spot at λ_{365} nm); ^1H NMR ([D_6]DMSO): δ = 7.25 (dd, 1 H, J = 7.4 Hz, 4'-H), 7.29 (dd, 1 H, J = 2.7 Hz, 1-H), 7.39 (d, 2 H, J = 7.4 Hz, 2'- and 6'-H), 7.45 (dd, 1 H, J = 2.7 Hz, 2-H), 7.61 (dd, 2 H, J = 8.4 Hz, 4-H and 5-H), 7.76 (d, 2 H, J = 7.4 Hz, 3'-H and 5'-H), 8.06 (d, 1H, J = 6.1 Hz, 7-H), 11.50 (bs, 1 H, NH), 11.98 (d, 1H, J = 5.7 Hz, NH); ^{13}C NMR ([D_6]DMSO): δ = 104.8, 111.9, 117.5, 119.2, 120.5, 123.5, 125.6, 126.3, 128.0, 128.9, 131.8, 135.5, 135.6, 137.3, 175 (CO); HRMS (ESI) calcd for $\text{C}_{17}\text{H}_{13}\text{N}_2\text{O}$ [$M+\text{H}$] $^+$ m/z 261.0975, found 261.1272; anal. calcd for $\text{C}_{17}\text{H}_{12}\text{N}_2\text{O}$: C 78.44, H 4.65, N 10.76, found: C 78.31, H 4.52, N 10.56.

3-Ethyl-8-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (34)—Light-brown solid; yield (93%); mp: 248–250 °C (EtOH); R_f = 0.45 (EtOAc/*n*-hexane 8:1, violet fluorescent spot at λ_{365} nm); ^1H NMR ([D_6]DMSO): δ = 1.40 (t, 3H, J = 7.3 Hz, CH_3), 4.32 (q, 2 H, J = 7.1 Hz, CH_2), 7.27 (dd, 1H, J = 7.4 Hz, 4'-H), 7.36 (dd, 1H, J = 8.5 Hz, 5-H), 7.40 (dd, 2 H, J = 7.6 Hz, 2'-H and 6'-H), 7.51 (d, 1 H, J = 2.7 Hz, 1-H), 7.60 (d, 1H, J = 2.7 Hz, 2-H), 7.78 (d, 2 H, J = 7.8 Hz, 3'- and 5'-H), 7.88 (d, 1 H, J = 8.5 and 0.6 Hz, 4-H), 8.07 (s, 1 H, 7-H), 12.02 (bs, 1 H, NH); ^{13}C NMR ([D_6]DMSO): δ = 16.3 (CH_3), 40.9 (CH_2), 104.2, 112.1, 115.7, 119.3, 120.6, 124.1, 126.4, 128.1, 128.3, 128.8, 131.1, 135.58, 135.7, 137.2, 175.8 (CO); HRMS (ESI) calcd for $\text{C}_{19}\text{H}_{17}\text{N}_2\text{O}$ [$M+\text{H}$] $^+$ m/z 289.1288, found 289.1266; anal. calcd for $\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}$: C 79.14, H 5.59, N 9.72, found: C 78.94, H 5.34, N 9.60.

3-Methylcyclopropyl-8-phenyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (35)—Grey solid; Yield 60 %; mp: 197–198 °C (70 % EtOH); R_f = 0.57 (EtOAc/*n*-hexane 8:2, violet fluorescent spot at λ_{365} nm); ^1H NMR ([D_6]DMSO): δ = 0.38 (m, 2 H, CH_2), 0.51 (m, 2 H, CH_2), 1.24 (m, 2 H, CH), 3.98 (d, 2H, J = 6.87 Hz, CH_2), 7.27 (dd, 1 H, J = 7.3 Hz, 4'-H), 7.38 (m, 3 H, 2'-, 6'- and 5-H), 7.55 (dd, 1H, J = 2.9 Hz, 2-H), 7.62 (dd, 1 H, J = 3.6 Hz, 1-H), 7.78 (d, 2H, J = 1.3 Hz, 5' and 3'-H), 7.91 (d, 1 H, J = 8.8 Hz, 4-H), 8.07 (d, 1 H, J = 6.3 Hz, 7-H), 12.0 (d, 1 H, J = 6.10 Hz, NH); ^{13}C NMR ([D_6]DMSO): δ = 4.1 ($2 \times \text{CH}_2$), 12.2 (CH), 50.2 (CH_2), 100.4, 104.1, 110.1, 114.8, 115.9, 118.9, 120.6, 124.0, 126.4, 128.1, 128.5, 128.9, 128.9, 130.4, 135.5, 137.3, 175.9 (CO); HRMS (ESI) calcd for $\text{C}_{21}\text{H}_{20}\text{N}_2\text{O}$ [$M+\text{H}$] $^+$ m/z 315.1445, found 315.1422; anal. calcd for $\text{C}_{21}\text{H}_{19}\text{N}_2\text{O}$: C 80.23, H 5.77, N 8.91, found: C 79.91, H 5.46, N 8.81.

3-Ethyl-8-(3-methoxyphenyl)-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (36)—Light-yellow solid; yield 60 %; mp: 262–264 °C (MeOH); R_f = 0.65 (EtOAc/*n*-hexane 9:1, violet fluorescent spot at λ_{365} nm); ^1H NMR ([D_6]DMSO): δ = 1.40 (t, 3H, J = 7.2 Hz, CH_3), 3.8 (s, 3 H, OCH_3), 4.32 (q, 2 H, J = 7.2 Hz, CH_2), 6.85 (m, 1 H, J = 1.5 Hz, 4'-H), 7.28 (d, 1 H, J = 8.3 Hz, 5-H), 7.33 (d, 1 H, J = 1.9 Hz, 6'-H), 7.36 (d, 1 H, J = 1.9 Hz, 5'-H), 7.43 (m, 1 H, J = 1.5 Hz, 2'-H), 7.45 (d, 1H, J = 2.9 Hz, 1-H), 7.61 (d, 1 H, J = 2.9 Hz, 2-H), 7.88 (dd, 1 H, J = 8.3 and 0.8 Hz, 4-H), 8.09 (s, 1H, 7-H), 12.00 (bs, 1H, NH); ^{13}C NMR ([D_6]DMSO): δ = 16.26 (CH_3), 40.9 (CH_2), 55.4 (OCH_3), 104.1, 111.9, 111.9, 114.6, 115.8, 120.3, 121.2, 124.0, 129.0, 129.1, 131.7, 135.4, 135.8, 138.6, 159.2, 175.8 (CO); HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{19}\text{N}_2\text{O}_2$ [$M+\text{H}$] $^+$ m/z 333.1511, found 333.1516; anal. calcd for $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_2$: C 75.45, H 5.70, N 8.80, found: C 75.18, H 5.35, N 8.71.

8-(4-Bromophenyl)-3-ethyl-3*H*-pyrrolo[3,2-*f*]quinolin-9(6*H*)-one (37)—Grey solid; yield 50 %; mp: 216–218 °C (MeOH/EtOH 80:20); R_f = 0.65 (EtOAc/*n*-hexane 9:1, violet fluorescent spot at λ_{365} nm); ^1H NMR ([D_6]DMSO): δ = 1.40 (t, 3 H, J = 7.2 Hz, CH_3), 4.32 (q, 2 H, J = 7.2 Hz, CH_2), 7.34 (d, 1 H, J = 3.2 Hz, 1-H), 7.36 (d, 1 H, J = 3.1 Hz, 2-H), 7.56 (dd, 2 H, J = 8.6 and J = 2.0 Hz, 2'- and 6'-H), 7.60 (d, 1 H, $J_{5,4}$ = 8.4 Hz, 5-H), 7.81 (dd, 2H, J = 8.6 and 1.97 Hz, 3' and 5'-H), 7.89 (dd, 1 H, $J_{5,4}$ = 8.4 and $J_{4,1}$ = 0.6 Hz, 4-H), 8.14 (s, 1 H, 7-H), 12.07 (bs, 1 H, NH); ^{13}C NMR ([D_6]DMSO): δ = 16.3 (CH_3), 40.9

(CH₂), 100.3, 110.3, 114.9, 118.9, 119.2, 119.3, 123.8, 128.5, 130.4, 130.8, 130.9, 132.3, 136.4, 153.8, 175.7 (CO); HRMS (ESI) calcd for C₁₉H₁₆BrN₂O [*M*+H]⁺ *m/z* 383.0538, found 332.0870; anal. calcd for C₁₉H₁₅BrN₂O: C 62.14, H 4.12, N 7.63, found: C 62.01, H 3.89, N 7.51.

Biology

Growth inhibitory activity—Human T-leukemia (Jurkat), promyelocytic leukemia (HL-60), chronic myelogenous leukemia (K562), myeloid leukemia (ML-2), and acute lymphoblastic leukemia (RS 4;11) cells, the latter with a t(4;11) translocation, were grown in RPMI-1640 medium (Gibco Milan, Italy). Human breast adenocarcinoma (MCF-7), cervix carcinoma (HeLa), ovarian cancer (IGROV), anaplastic thyroid (ARO), hepatoma (HepG2), and colon adenocarcinoma (HT-29) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with penicillin G (115 U mL⁻¹; Gibco), streptomycin (115 mg mL⁻¹; Invitrogen, Milan, Italy) and 10 % fetal bovine serum (Invitrogen). Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 mL complete medium containing 8×10^3 cells. The plates were incubated at 37 °C in a humidified incubator containing 5% CO₂ for 18 h. At this time, the initial medium was removed from each well, and drug solutions (100 µL each), dissolved in complete medium, at various concentrations, was added to each well and incubated at 37°C for 72 h. Cell viability was assayed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) test as previously described.[25] The GI₅₀ value is defined as the compound concentration required to inhibit cell proliferation by 50 %.

Effects on tubulin polymerization and on colchicine binding to tubulin—Bovine brain tubulin was purified as described previously. To evaluate the effect of the compounds on tubulin assembly in vitro, various concentrations were pre-incubated with 10 mM tubulin in glutamate buffer at 30 °C and then cooled to 0 °C. After addition of GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30°C, and the assembly of tubulin was observed turbidimetrically. The IC₅₀ value is defined as the compound concentration required to inhibit the extent of assembly by 50 % after incubation for 20 min. The capacity of the test compounds to inhibit colchicine binding to tubulin was measured as described,[8,9] except that the reaction mixtures contained 1 mM tubulin, 5 mM [³H]colchicine, and 1 mM test compound.

Flow cytometric analysis of cell-cycle distribution and apoptosis—For flow cytometric analysis of DNA content, 5×10^5 Jurkat cells in exponential growth were treated with various concentrations of the test compounds for 24 and 48 h. After an incubation period, the cells were collected, centrifuged, and fixed with ice-cold ethanol (70 %). The cells were then treated with lysis buffer containing RNase A and Triton X-100 (0.1 %), and then stained with PI. Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter). DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems, CA, USA).

Annexin V assay—Surface exposure of PS by apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Beckman Coulter, USA) by adding annexin V–FITC to cells according to the manufacturer's instructions (annexin V Fluos, Roche Diagnostics). The cells were simultaneously stained with PI. Excitation was set at λ 488 nm, and the emission filters were set at λ 525 and 585 nm.

Assessment of mitochondrial changes—The mitochondrial membrane potential was measured with the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, USA), as described.

[25] Briefly, after various times of treatment, the cells were collected by centrifugation and resuspended in Hank's Balanced Salt Solution (HBSS) containing JC-1 at a concentration of 1 mM. The cells were then incubated for 10 min at 37 °C, centrifuged, and resuspended in HBSS. The production of ROS was measured by flow cytometry using HE (Molecular Probes) and H₂-DCFDA (Molecular Probes).

After various treatment times, the cells were collected by centrifugation and resuspended in HBSS containing the fluorescence probes HE and H₂-DCFDA at concentrations of 2.5 and 0.1 mM, respectively. The cells were then incubated for 30 min at 37°C, centrifuged, and resuspended in HBSS. The fluorescence was recorded directly with the flow cytometer using an excitation wavelength of λ 488 nm and emission at λ 585 and 530 nm for HE and H₂-DCFDA, respectively.

Caspase-3 assay—Caspase-3 activation in Jurkat cells was evaluated by flow cytometry using a human active caspase-3 fragment antibody conjugated with FITC (BD Pharmingen) as described.[26] Briefly, after various incubation times in the presence of test compounds, the cells were collected by centrifugation and resuspended in Cytotfix™ (BD Pharmingen) buffer for 20 min, washed with Perm/Wash™ (BD Pharmingen) and then incubated for 30 min with the antibody. After this period the cells were washed and analyzed by flow cytometry. The results are expressed as a percentage of caspase-3 active fragment positive cells.

Flow cytometric analysis of Bcl-2 expression—Bcl-2 expression was evaluated in Jurkat cells by flow cytometry using a monoclonal antibody conjugated with FITC (DAKO). Briefly, after various incubation times in the presence of test compounds, the cells were collected by centrifugation and permeabilized using a Fix&Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA, USA). The cells were then labeled with Bcl-2 antibody, washed, and analyzed by flow cytometry.

Western blot analysis—Jurkat cells were incubated in the presence of test compounds and, at various time points, were collected, centrifuged, and washed twice with ice-cold PBS. The pellet was then resuspended in lysis buffer. After the cells were lysed on ice for 30 min, lysates were centrifuged at 15 000 g at 4°C for 10 min. The protein concentration in the supernatant was determined using BCA protein assay reagents (Pierce, Italy). Equal amounts of protein (20 μ g) were resolved by SDS-PAGE (12 % acrylamide) and transferred to PVDF Hybond-p membrane (GE Healthcare). Membranes were blocked with I-block (Tropix) overnight, under rotation at 4 °C. Membranes were then incubated with a primary antibody against the cleaved fragment of PARP (rabbit, 1:1000, Cell Signaling), or β -actin (mouse, 1:10 000, Sigma) for 2 h at room temperature. Membranes were next incubated with peroxidase-labeled goat anti-rabbit IgG (1:100 000, Sigma) or peroxidase-labeled goat anti-mouse IgG (1:100 000, Sigma) for 60 min. All membranes were visualized using ECL Advance (GE Healthcare) and exposed to Hyper film MP (GE Healthcare). To ensure equal protein loading, each membrane was stripped and re-probed with anti- β -actin antibody.

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Figure 1.
3*H*-Pyrrolo[3,2-*f*]quinolin-9(6*H*)-ones; SARs are described in the text.

Figure 2. Structures of compounds **38**, [3] CA-4, and colchicine taken as references in the assays of antiproliferative activity, inhibition of tubulin polymerization and colchicine binding, respectively.

Figure 3.

Effects of compounds A) **11**, B) **12**, C) **13**, and D) **22** on cell cycle distribution and G₂/M arrest in Jurkat cells after 24 h incubation (■: G₁, ●: G₂/M, ▲: S). Cells were treated with various concentrations of test compounds (0.015–1.0 mM). The cells were then fixed and stained with propidium iodide (PI) to analyze DNA content by flow cytometry. Data are expressed as the mean ±SEM for two independent experiments.

Figure 4. Flow cytometric analysis of apoptotic cells after treatment of Jurkat cells with compounds **11** (■), **12** (●), **13** (▲), or **22** (▼). After treatment for A) 24 or B) 48 h, cells were harvested and labeled with annexin V-FITC and PI and then analyzed by flow cytometry. The data are expressed as the mean percentage of annexin-V-positive cells $\pm$ SEM for three independent experiments.

Figure 5.

Assessment of mitochondrial dysfunction after treatment with compounds **11** or **12**. A) Induction of loss of mitochondrial membrane potential in Jurkat cells after 24 and 48 h incubation with **11** and **12** at 0.1 and 0.5 mM. Cells were stained with the fluorescent probe JC-1 and analyzed by flow cytometry. B) and C) Mitochondrial production of ROS in Jurkat cells after 24 and 48 h incubation with **11** and **12**. Cells were stained with B) HE or C) H₂-DCFDA and analyzed by flow cytometry. The data are expressed as a percentage of the fluorescence intensity relative to untreated control and represent the mean $\pm$ SEM of three independent experiments.

Figure 6.

Caspase-3 activity induced by compounds **11** and **12**. Jurkat cells were incubated in the presence of A) **11** or B) **12** at 0.1 and 0.5 mM. After treatment for 48 h, cells were harvested and stained with an anti-human active caspase-3 fragment monoclonal antibody conjugated with FITC. Data obtained by flow cytometry analysis are expressed as the percentage of caspase-3 active fragment positive cells. Data are expressed as the mean $\pm$ SEM of three independent experiments.

Figure 7.

Western blot analysis of PARP and flow cytometric analysis of Bcl-2 expression. A) Jurkat cells were treated with compounds **11** and **12** at the indicated concentrations for 24 and 48 h. The cells were then subjected to immunoblot analysis as described in the Experimental Section. B) Cells were treated as above, and after permeabilization were labeled with a Bcl-2 FITC-conjugated antibody. Shown are representative histograms after 24 h treatment with compound **11** at the indicated concentrations. C) Bcl-2 expression in Jurkat cells after treatment with compounds **11** and **12** at the indicated concentrations for 24 and 48 h. Data are expressed as the mean $\pm$ SEM of two independent experiments.

Scheme 1.

Synthesis of pyrroloquinolinone derivatives **11–13**. *Reagents and conditions*: a) *t*BuOK, benzene, 80 °C, 6 h; b) NaH, ethyl acrilate or trimethoxybenzyl chloride, DMF, 70 °C, 4 h and 24–48 h, respectively; c) H₂, Pd/C (10 %), EtOH, room temperature, 3 h; d) ethyl benzoylacetate or **1 a**, EtOH, Drierite, reflux, 24 h; e) Ph₂O, reflux, 30 min.

Scheme 2.

Synthesis of 7-phenylpyrroloquinolinone derivatives **17**, **21**, and **22**. *Reagents and conditions:* a) POCl_3 , DMF, reflux, 3–5 h; b) and f) H_2 , Pd/C (10 %), EtOAc, 40 °C, 10–24 h; c) and g) ethyl benzoylacetate, EtOH, Drierite, reflux, 24 h; d) and h) Ph_2O , reflux, 30 min; e) $\text{NH}_2\text{OH}\cdot\text{HCl}$, pyridine, 80 °C, 2 h, SeO_2 , Na_2SO_4 , DMF, 80 °C, 2.5 h; i) $\text{CH}_3\text{COOH}/\text{H}_2\text{SO}_4$, H_2O , reflux, 16 h.

Scheme 3.

Synthesis of 8-phenylpyrroloquinolinone derivatives **33–37**. *Reagents and conditions:* a) NaH, ethyl formate, room temperature, 24 h; b) EtOH, room temperature, 10–15 h; c) Ph₂O, reflux, 15 min.

Table 1
In vitro inhibitory effects on cell proliferation by compounds **11–13**, **17**, **20**, **21**, **33–37**, and **38**.

Compd	HT-29	ARO	Hep G2	IGROV	MCF-7	HeLa	HL-60	ML-2	K562	RS 4;11	Jurkat
11	0.48 ± 0.08	0.55 ± 0.15	0.52 ± 0.10	0.62 ± 0.15	0.58 ± 0.18	0.45 ± 0.10	0.002 ± 0.0001	0.005 ± 0.002	0.19 ± 0.06	0.002 ± 0.0004	0.0005 ± 0.00001
12	0.45 ± 0.10	0.58 ± 0.15	0.95 ± 0.15	0.85 ± 0.20	1.40 ± 0.50	0.40 ± 0.05	0.002 ± 0.0001	0.01 ± 0.002	0.03 ± 0.007	0.001 ± 0.0001	0.0003 ± 0.00001
13	0.85 ± 0.14	0.45 ± 0.07	0.50 ± 0.05	0.65 ± 0.15	0.85 ± 0.15	0.40 ± 0.05	0.03 ± 0.002	0.3 ± 0.04	0.24 ± 0.03	0.04 ± 0.007	0.05 ± 0.004
17	>50	>50	5.8 ± 1.1	>50	>50	4.5 ± 0.8	17.5 ± 2.3	14.4 ± 2.3	27.3 ± 0.34	6.5 ± 1.0	8.0 ± 1.2
21	>50	>50	>50	>50	>50	>50	0.21 ± 0.01	0.55 ± 0.03	0.46 ± 0.008	0.13 ± 0.02	0.3 ± 0.01
22	>50	>50	>50	>50	>50	>50	0.32 ± 0.03	0.09 ± 0.02	1.7 ± 0.5	0.03 ± 0.002	0.05 ± 0.006
33	>50	>50	>50	ND	ND	>50	>50	>50	>50	ND	ND
34	>50	>50	>50	ND	ND	>50	>50	>50	>50	ND	ND
35	>50	>50	>50	ND	ND	>50	>50	>50	>50	ND	ND
36	>50	>50	>50	ND	ND	>50	>50	>50	>50	ND	ND
37	>50	>50	>50	ND	ND	>50	>50	>50	>50	ND	ND
38[3]	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.01 ± 0.008	0.0005 ± 0.00002	0.001 ± 0.0003	0.001 ± 0.0001	0.002 ± 0.0003	0.0005 ± 0.00002

[a] GI₅₀: compound concentration required to inhibit tumor cell proliferation by 50 %; data are expressed as the mean ± SE from the dose-response curves of at least three independent experiments; ND: not determined. See text for details of the cell lines examined; the first six cell lines listed are derived from human solid tumors, the last five are from human leukemias.

Table 2Inhibition of tubulin polymerization and colchicine binding by compounds **11–13**, **22**, **38**, and CA-4.

Compd	Tubulin Assembly ^[a] IC ₅₀ [μM]	Colchicine Binding ^[b] Inhibition [%]		
		1 μM	5 μM	50 μM
11	0.75 ± 0.04	44 ± 6	75 ± 4	ND
12	1.4 ± 0.2	ND	30 ± 4	56 ± 2
13	2.4 ± 0.4	ND	23 ± 6	65 ± 0.3
22	>40	ND	ND	ND
38	0.57 ± 0.02	34 ± 2	73 ± 0.7	ND
CA-4	1.2 ± 0.01	84 ± 3	98 ± 0.3	ND

^[a] Inhibition of tubulin polymerization; tubulin was present at 10 μM; error values are ± SD.

^[b] Inhibition of [³H]colchicine binding; tubulin and colchicine were present at 1 and 5 μM, respectively, and the tested compounds were present at 1, 5, or 50 μM, as indicated; ND: not determined.