Synthesis and in Vitro and in Vivo Antitumor Activity of 2-Phenylpyrroloquinolin-4-ones

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In our search for potential new anticancer drugs, we designed and synthesized a series of tricyclic compounds containing the antimitotic 2-phenylazaflavone chromophore fused to a pyrrole ring in a pyrroloquinoline structure. Compounds **8**, **18**, **19**, **22**, **23**, **25** and **26**, when tested against a panel of fourteen human tumor cell lines, showed poor in vitro cytotoxic activity, whereas **20**, **21** and **24** showed significant activity (IC_{50} 0.7 to 50 μM). Steroid hormone-sensitive ovary, liver, breast and adrenal gland adenocarcinoma cell lines displayed the highest sensitivity (IC_{50} 0.7 to 8 μM). Compound **24** blocked cells in the G_2/M phase of the cell cycle and induced a significant increase in apoptotis. Compounds **20**, **21** and **24** proved to alter microtubule assembly and stability, displaying a cytoplasmic microtubule network similar to that caused by Vincristine. In vivo, administration of compound **24** to Balb/c mice inhibited the growth of a syngenic hepatocellular carcinoma.

Introduction

In the field of anticancer drugs, microtubules are still a valid target, and it is probable that antimitotic drugs will continue to be important chemotherapeutic agents, especially as more selective approaches are developed.[1] Growing understanding of their mechanisms of action, and the synergy shown in combination therapies, together with the desire to obtain orally available analogues and the need to overcome neurotoxicity and the development of antimitotic resistance have all produced ever increasing interest, aimed at discovering more effective agents and therapeutic strategies.

Most of the antimitotic compounds used in clinical practice are either naturally occurring molecules or their synthetic analogues, such as vinca alkaloids, taxol, colchicine, podophyllotoxin and combretastatin A-4.[2] Interestingly, it has recently been reported that some natural flavonoids (Figure 1, 2-phenyl-benzopyran-4-ones **I**) also exert potent cytotoxic activity against several cancer cell lines, both in vitro and in vivo by interfering with tubulin polymerization.[3,4] However, since these natural compounds interact with various biological targets,[5−8] their antitumor properties may be the result of multiple activities antioxidant,[5] antimutagenic,[5,40] pro-apoptotic,[9] antitopoisomerase[10] and antiestrogenic.[11−16]

To enhance specifically the antimitotic activity of flavonoid compounds, some authors have synthesized new azaflavones[2,17−19] (Figure 1, 2-phenylquinolin-4-ones, **II**) and a series of bioisosteric bicyclic diaza analogues, quinazolinones[2] (Figure 1, **III**) and naphthyridinones[2,20−22] (Figure 1, **IV**). All these classes of compounds have strong antimitotic effects and appear to interact specifically with the colchicine binding site on β tubulin.[19,23] SAR and QSAR studies have also indicated very stringent structural and spatial requirements for the antimitotic activity of 2-phenylazaflavones, indicating that specific substitutions on the side phenyl ring, together with others on the quinolinone ring, may be important for their antiproliferative activity.[19,24]

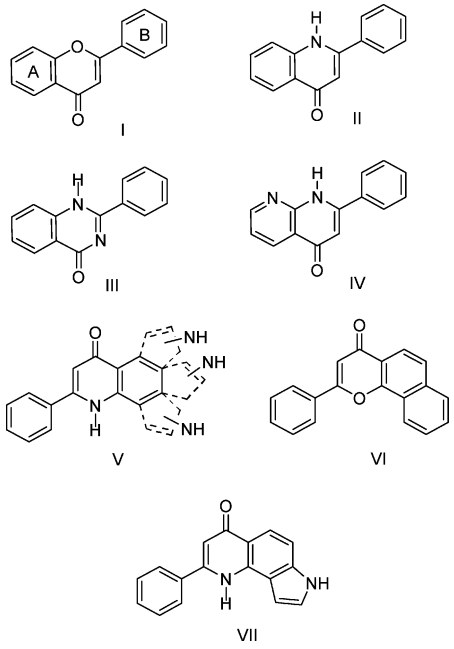

Figure 1.

As part of our search for synthetic pyrroloquinoline-derivative compounds with antitumor activity,[25,26] we designed and synthesized a series of 2-phenylpyrrolo-

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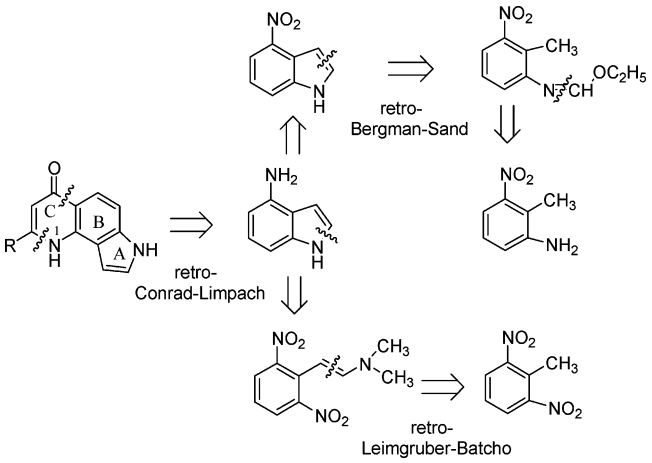
Scheme 1. Retrosynthetic Routes

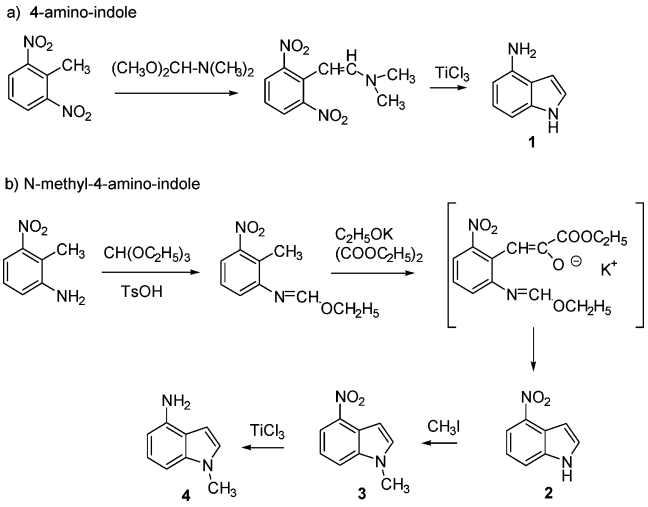


quinolinones (Figure 1, **V**): these may be viewed as the antimitotic 2-phenylazaflavone pharmacophore modified by fusion to a pyrrole ring. This structural modification, suitable for further substitutions, in view of controlled phamacodynamic and pharmacokinetic testing, will be useful for more detailed SAR information about this class of molecules. Although we expected that the new compounds would exert antimitotic activity, it should be recalled that enlargement of a bicyclic structure to a planar tricyclic one may result in additional mechanisms of action, such as antitopoisomerase I and II activity. Furthermore, looking at the structural similarity of the newly synthesized 2-phenyl-1*H*,7*H*-pyrrolo-[2,3-*h*]quinolin-4-ones (Figure 1, **VII**) with flavones and 7,8-benzoflavones[6] (Figure 1, **VI**), a certain interaction with estrogen aromatase enzyme may also be hypothesized. This paper reports the synthesis of nine new tricyclic derivatives, characterized by the pyrrolo[2,3-*h*]quinolin-4-one nucleus bearing a methyl group or a variously substituted phenyl ring in position 2. All compounds were evaluated for their in vitro antiproliferative activity on human tumor cell lines, and in vivo on a syngenic hepatocellular carcinoma in Balb/c mice (**24**). In addition, to obtain some preliminary insights on the mechanism(s) of action, their effects on cell cycle and microtubule dynamics, programmed cell death (apoptosis) and aromatase and topoisomerase I and II enzymatic activity were also examined.

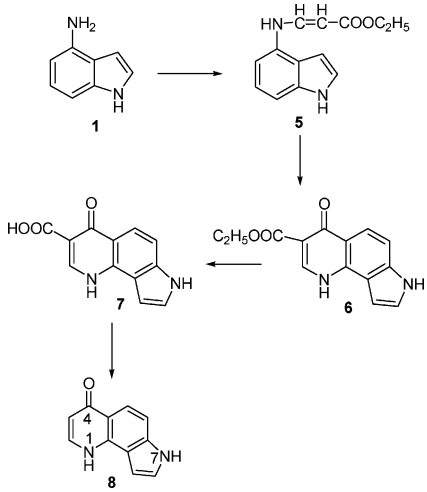
Results and Discussion

Chemistry. On the basis of our previous experience with pyrroloquinoline nucleus synthesis[25,26] and in view of the retrosynthetic pathways briefly shown in Scheme 1, 4-aminoindole was chosen as the starting material, leading to the tricyclic structure of 2-substituted-pyrrolo[2,3-*h*]quinolin-4-one by a retro-Conrad−Limpach[27] disconnection at the N-1 and C-2 bonds in the C ring. As 4-aminoindole or its precursor 4-nitroindole are commercially available but extremely expensive, we prepared them ourselves. 4-Aminoindole[28] may be disconnected to a vinyl intermediate and the latter brought to 2,6-dinitrotoluene via a retro-Leimgruber−Batcho reaction. Leimgruber−Batcho synthesis[29] is a two-step method providing indoles which are only substituted on the benzene ring. The 4-nitroindole precursor, useful for the synthesis of N[7]-alkylated tricyclic derivatives, can

Scheme 2. Synthesis of Aminoindoles



Scheme 3. Synthetic Route to 1*H*,7*H*-Pyrrolo [2,3-*h*]quinoline-4-one (**8**)

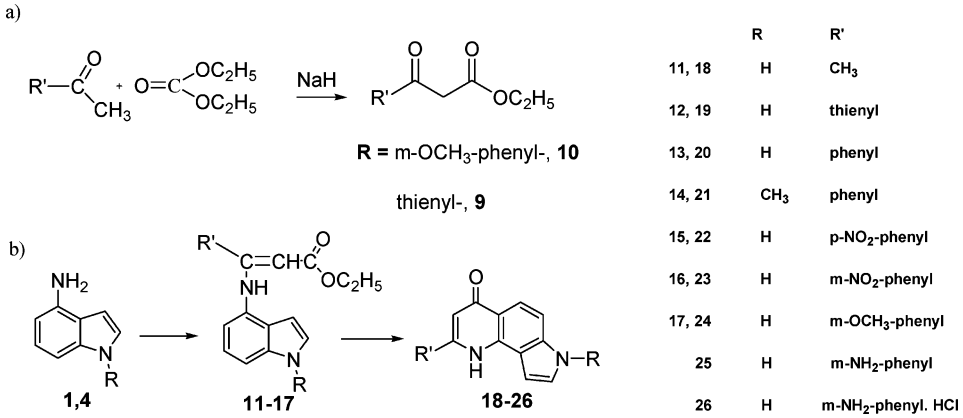


be disconnected to *o*-nitrotoluidine by two retrosynthetic steps of the Madelung indole synthesis variation reported by Bergman and Sand.[30]

Scheme 2 shows the preparation of aminoindoles **1** and **4**. For compound **1**, 2,6-dinitrotoluene, treated with dimethylformamide dimethylacetal (DMFDMA) in *N*,*N*-dimethylformamide (DMF), produced the dimethylaminostyrene derivative in an almost quantitative yield already after only 4 h. This was subjected to reductive cyclization with $TiCl_3$ to give 4-aminoindole: we chose this reagent, because it provided a higher yield with respect to other methods (H_2, Pd/C) over a shorter reaction time (minutes instead of hours).[28] For compound **4**, we first obtained 4-nitroindole from 2-methyl-3-nitroaniline, which was reacted with triethyl orthoformate in the presence of *p*-toluensulfonic acid to give the imidate ester. Treatment of the latter with diethyloxalate and potassium ethoxide produced 4-nitroindole **2**.[30,31] As regards experimental procedures and reaction products **1** and **2**, no evident differences were noted compared with those previously reported.

Next, **2** was alkylated to the N^1-methyl derivative **3** by CH_3I and KOH in acetone at room temperature, which turned out to be a more advantageous method[32] than the usual one (CH_3I, NaH).[33,34] Again, the following

Scheme 4[a]



	R	R'
11, 18	H	CH_3
12, 19	H	thienyl
13, 20	H	phenyl
14, 21	CH_3	phenyl
15, 22	H	p-NO_2-phenyl
16, 23	H	m-NO_2-phenyl
17, 24	H	m-OCH_3-phenyl
25	H	m-NH_2-phenyl
26	H	m-NH_2-phenyl. HCl

[a] (a) Synthesis of β-ketoesters **9** and **10**. (b) General route to 2-substituted-pyrroloquinolinones.

Table 1. In Vitro Cytotoxic Activities of 2-Substituted-pyrroloquinolin-4-ones **8**, **18**−**26** Compared with Vincristine Sulfate Salt

	cytotoxicity, IC_{50} (μM)					
compd	Aro	C8305	A-172	Mia Paca 2	PT45	HT-29
8	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100
19	80	100	>100	>100	100	>100
20	8	10	100	4	100	60
21	5	10	>100	8	100	50
22	>100	>100	>100	>100	>100	>100
23	80	>100	>100	>100	>100	>100
24	4	10	>100	5	100	50
25	70	80	>100	>100	>100	>100
26	>100	>100	>100	>100	>100	>100
vincristine sulfate salt	<0.0001	0.1	>100	0.01	0.1	0.1

Table 2. In Vitro Cytotoxic Activities of 2-Substituted-pyrroloquinolin-4-ones **8**, **18**−**26** Compared with Vincristine Sulfate Salt

	cytotoxicity, IC_{50} (μM)							
compd	Hep 3B	Hep G2	PLC/PRF/5	SW13	NCI−H295	Ovcar-3	Igrov-1	MCF-7
8	>100	>100	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100	>100	>100
19	50	10	80	50	40	20	15	30
20	4	4	8	3	8	2	5	4
21	1	1	7	0,9	2	2	3	4
22	30	100	>100	50	80	50	60	60
23	50	>100	>100	60	60	50	80	70
24	2	2	7	0,7	3	1	3	1
25	>100	50	50	10	50	30	30	50
26	>100	>100	>100	>100	>100	>100	>100	>100
vincristine sulfate salt	0.1	0.1	0.1	0.001	0.1	0.01	0.01	0.01

reduction reaction was performed with $TiCl_3$, leading to almost pure *N*-methyl-4-aminoindole (**4**) in good yields.

Synthesis of unsubstituted 1*H*,7*H*-pyrrolo[2,3-*h*]-quinolin-4-one (**8**) was carried out with a four-step pathway (Scheme 3). Starting from 4-aminoindole, we constructed the tricyclic compound **8** by means of the Gould−Jacob reaction,[35] a method used to obtain the quinolin-4-one nucleus which may subsequently lead to 2,3-unsubstituted derivatives. Condensation of **1** with diethyl ethoxymethylenemalonate to **5**, thermal cyclization to **6**, ester saponification to **7** and a final decarboxylation reaction produced pyrroloquinolinone **8**.

The syntheses of 2-substituted pyrroloquinolin-4-ones are outlined in Scheme 4.

(a) Ethyl thiophencarbonyl acetate[20] **9** and ethyl *m*-methoxybenzoyl acetate[36] **10** were prepared, according to a method reported in the literature,[37] by condensation of 2-acetylthiophene and *m*-methoxyacetyl benzene with anhydrous diethyl carbonate in the presence of sodium hydride.

(b) Following Conrad−Limpach synthesis[27] of the quinoline nucleus, 4-aminoindoles **1**, **4** were condensed with β-keto-esters **a**−**d** and **9**, **10** to give enamine derivatives **11**−**17**, which were subjected to thermal cyclization to give final compounds **18**−**24**.

m-Nitro derivative **23** was also reduced by $TiCl_3$ to the corresponding amino compound **25** and then to the more stable dihydrochloride **26**.

Biology. In Vitro Cytotoxic Activity of 2-Phenylpyrroloquinolin-4-ones. Tables 1 and 2 show the in vitro cytotoxic activity of compounds **8**, **18**−**26**, tested against vincristine sulfate salt as reference on a panel of 14 human tumor cell lines, derived largely from solid tumors including liver, pancreas, colon, central nervous system, thyroid, adrenal gland, ovary and breast.

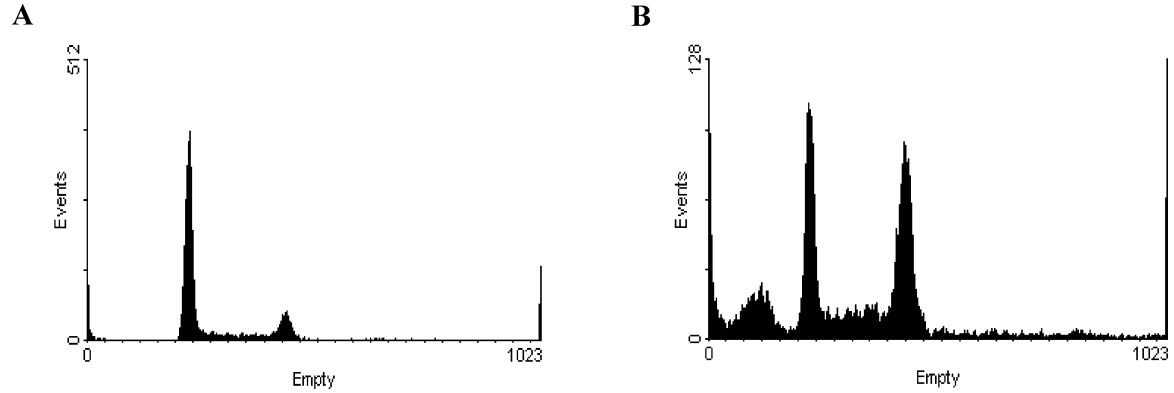


Figure 2. Effects of compound **24** on cell cycle dynamics as determined by Flow Cytometry. Figure 2A. DNA fluorescence flow cytometric profiles of propidium iodide-stained ovary carcinoma Ovcar-3 cells before treatment. Figure 2B. As above, after 24 h incubation with compound **24** (5 μM).

As expected, compounds **8** and **18**, carrying respectively a hydrogen and a methyl group in position 2, were devoid of activity, confirming the importance of a 2-phenyl ring for quinolin-4-one moiety cytotoxicity.[19] For 2-phenyl derivatives, compounds **19**, **22**, **23**, **25** and **26** had negligible cytotoxic effects against all cell lines tested, as their IC_{50} were generally above 50 μM. This is consistent with literature[17] data, which reports a loss of activity for compounds carrying strong electron-withdrawing groups such as the NO_2 group (**22**, **23**) or electron-donating groups, especially if they are inserted in ortho and para positions, whereas the presence of a free phenyl ring or one substituted with 3′-OCH_3 enhances cytotoxic activity, as observed for compounds **20**, **21** and **24**.

Since compounds **20**, **21** and **24** were 50-fold more active against hormone-responsive cell lines, like liver, ovary, breast and adrenal gland adenocarcinomas (IC_{50} 0.7 to 8 μM, Table 2) than against nonresponsive ones (cf. IC_{50} values in Tables 1 and 2), this result revealed a considerable selective effect, which 2-phenylazaflavones and their analogues do not show.[18]

We may therefore state that the fusion of a pyrrole ring at the 7,8 position of the 2-phenylquinolin-4-one pharmacophore leads to molecules which retain their cytotoxic activity and may also interfere with estrogen-supported tumor cell growth.

For insights into the mechanism(s) of action of these new compounds, we assessed the effects of the most cytotoxic **20**, **21** and **24**, on cell cycle and microtubule dynamics, on induction apoptosis and on CYP19 (aromatase) and topoisomerase I and II enzymatic activity.

Antimitotic Activity. On the basis of the chemosensitivity profiles of compounds **20**, **21** and **24**, hormone-sensitive cell lines were selected as prototypes, to explore their mode of action on induced cell death. Changes in SW13, HepG2, MCF-7 and Ovcar-3 cellular proliferation (DNA content and distribution) during treatment with compound **24**, used for the assays, were monitored by flow cytometry over a period of 48 h, a sufficient time interval for these cell lines to complete their cycle (data not shown).

As shown in Figure 2, compound **24** induced a significant increase in cells blocked in the G_2/M phase of the cell cycle: the percentage of cells found in the G_2/M phase was 38.87% (Figure 2B), compared with 16.84% for untreated cells (Figure 2A) after 24 h of

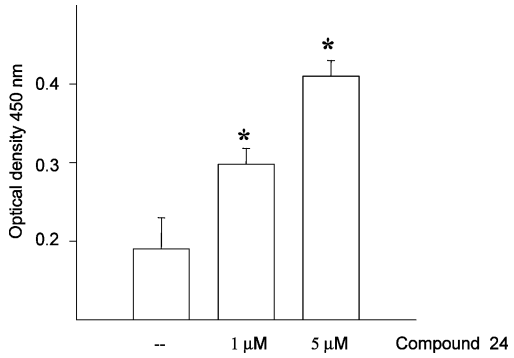


Figure 3. Apoptosis cell death. Ovcar-3 cells were incubated for 24 h with compound **24** (1−5 μM) and presence of histone-associated DNA fragments in cytoplasm was quantified by ELISA assay. Results are expressed as means ± SE (optical density values at 420 nm) from two different experiments carried out in triplicate. *P < 0.01 vs control (untreated cells).

treatment. The capacity of a drug to block cells in the G_2/M phase of the cell cycle is consistent with disruption of the mitotic spindle by interaction with the protein tubulin. Drugs which interfere with tubulin, and therefore with cell division, can be shown to cause this effect by flow cytometry.[38]

Furthermore, in all hormone-sensitive cell lines tested, a sub-G_0 peak (10.73%, Figure 2B) was observed after 24 h, indicating the presence of apoptotic cells with fragmented DNA.[39] Similar profiles (data not shown) were obtained for cells exposed to methotrexate, a well-known pro-apoptotic agent. To confirm the potential pro-apoptotic effect of 2-phenylpyrroloquinolin-4-ones on hormone-sensitive cell lines, we quantified the presence of histone-associated-DNA complexes in the cytoplasm, a well-accepted marker of apoptosis, in cells exposed for 24−48 h to 1−5 μM of compound **24**. As shown in Figure 3, incubation of Ovcar-3 cells with compound **24** for 24 h induced a significant increase in histone−DNA complexes in the cytoplasm, indicating induction of cell death via an apoptotic pathway.

To further explore the antimitotic activity of compounds **20**, **21** and **24**, their effects on microtubule dynamics were evaluated in Ovcar-3 (Figure 4A) and MCF-7 (Figure 4B) cell lines, stained with TRITC-labeled anti-β-tubulin antibody. As shown in Figure 4, the three compounds were able to destabilize microtubules both in the interphase and during mitotic division, causing a loss of the normal microtubule

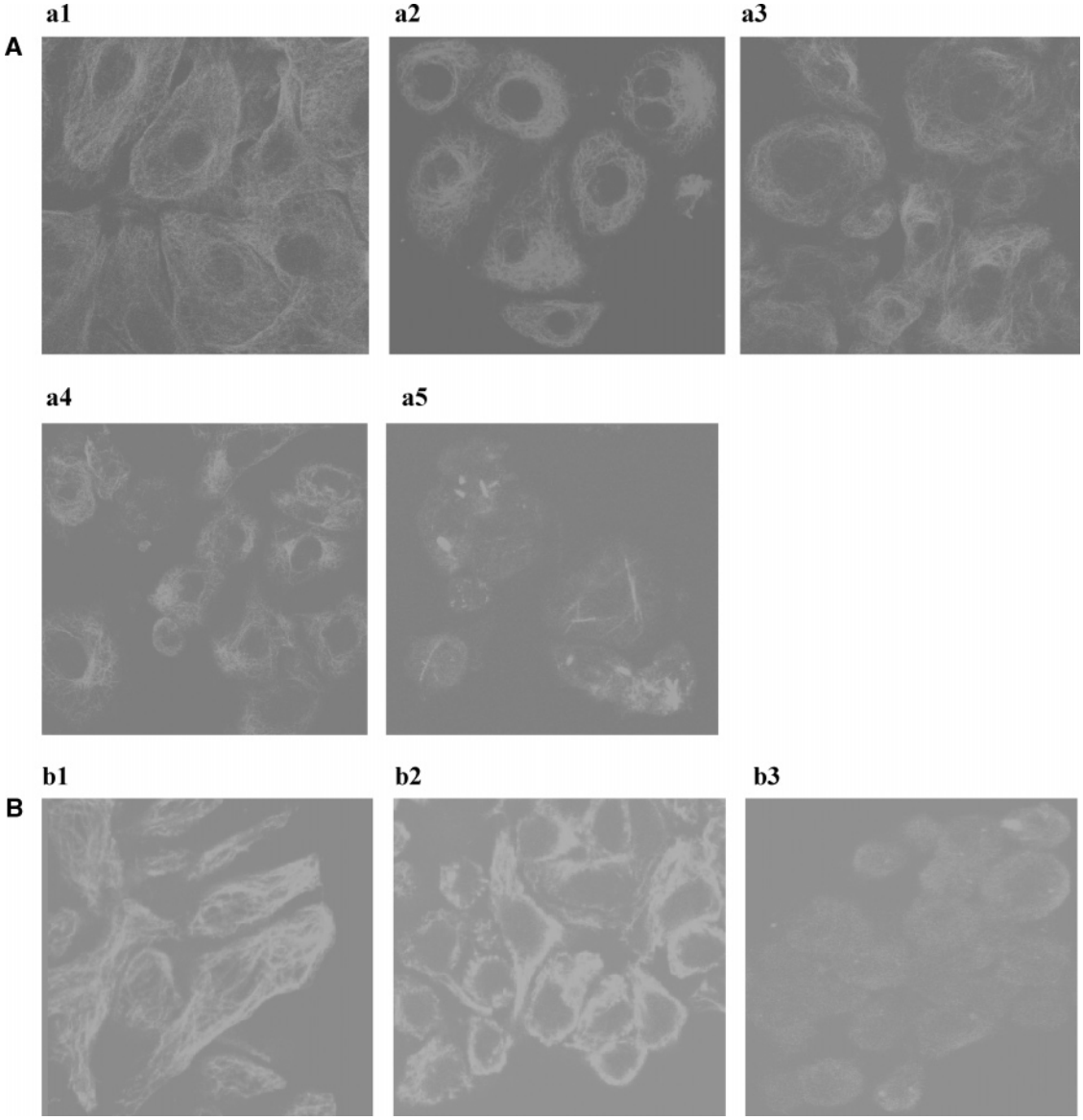


Figure 4. Immunofluorescence images of Ovcar-3 and MCF-7 cells revealed by staining with TRITC-labeled anti-β-tubulin antibody. Ovcar-3 (Figure 4A) and MCF-7 (Figure 4B) cells were incubated for 18 h with medium drug-free (**a1**, **b1**), compounds (5 μM) **20** (**a2**, **b2**), **21** (**a3**), **24** (**a4**) and vincristine (5 μM) (**a5**, **b3**). Cells were then fixed, stained for β-tubulin to visualize mitotic spindle and interphase microtubules and analyzed by fluorescence microscopy. Treatment with compound **20** on Ovcar-3 (**a2**) and MCF-7 (**b2**) cell lines, and compounds **21**, **24** on Ovcar-3 cells (**a3** and **a4**), caused loss of the normal microtubule network, like that of vincristine (**a5**, **b3**) but much less evident.

network similar to that induced by vincristine but much less evident. In particular, after treatment with compounds **20**, **21** and **24** (5 μM) for 18 h, the microtubule network appeared to be "rarefied" and fragmented.

The ability of the 2-phenylpyrroloquinolinone derivatives to induce the mitotic arrest and apoptosis is most likely attributable to their antimicrotubule activity.

Inhibition of Aromatase. To verify aromatase activity, initially hypothesized for the structural similarity of pyrrolo[2,3-*h*]quinolinones with the potent aromatase inhibitors flavones and 7,8-benzoflavones[6,41] (Figure 1, VI) and then reinforced by observed selectivity, the effects of compounds **20**, **21** and **24** on enzyme activity were investigated by applying the tritiated water release assay[42] to cell line H295R. Figure 5 shows that compounds **20**, **21** and **24** inhibited enzyme activity in a dose-dependent way, both in basal conditions (Figure 5A) and after induction with forskolin (Figure 5B), a substance which up-regulates CYP19 activity and gene transcripts by adenylate cyclase activation.[43] Limited exposure (3 h) of these compounds exerted good inhibitory activity although to a lesser extent than letrozole, one of the most potent aromatase inhibitors currently used in therapy.[44]

Inhibition of Topoisomerases. We also investigated the ability of **20**, **21** and **24** to inhibit the catalyzed reactions of nuclear enzymes topoisomerase I (relaxation) and topoisomerase II (decatenation). In detail, none of the tested compounds appeared able to interfere with the relaxation ability of topoisomerase I (data not shown). The effects on the decatenation reaction catalyzed by topoisomerase II are shown in Figure 6. In particular, **20** was able to inhibit significantly the formation of decatenated topoisomerase II products. In the same experimental conditions, **24**

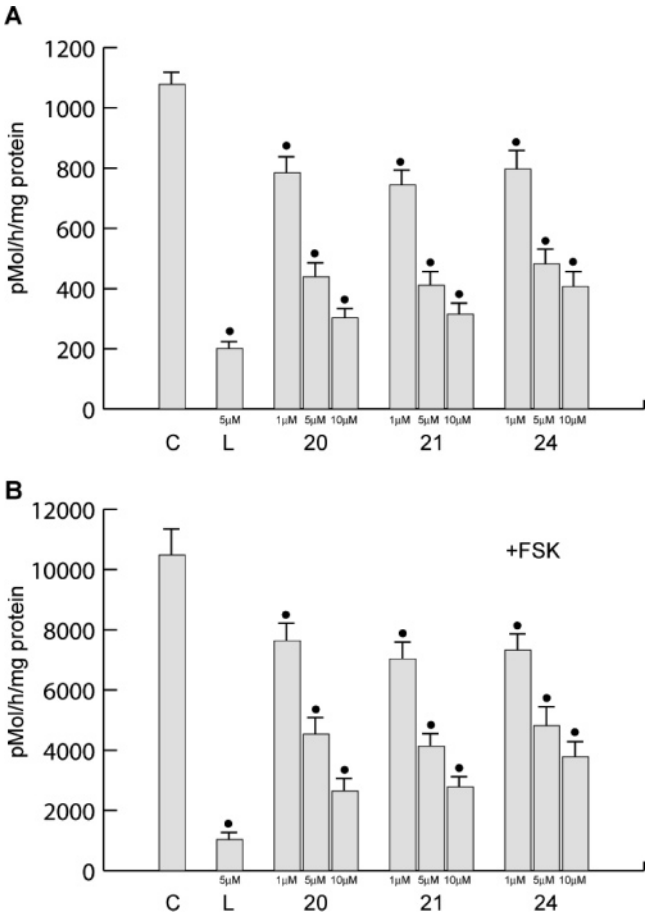


Figure 5. Inhibition of aromatase activity in H295R cells by compounds **20**, **21** and **24**. H295R cells were cultured for 24 h in DMEM-F12 in absence (A) or in the presence (B) of FSK (25 μM). One hour before addition of substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione (0.5 μM), compounds **20**, **21** and **24** and letrozole (L) were added to cells at concentrations shown. After a further 2-h exposure, aromatase activity was assessed using modified tritiated water method. Results are expressed as pmoles [^{3}H]H_2O released per hour and were normalized for mg protein (pmol/h/mg protein). Values represent means ± SEM of three different experiments, each performed in triplicate. (●) $P < 0.01$ compared with control (C) cells.

induced weak inhibition of enzymatic activity; **21** appeared unable to exert any detectable effect.

In Vivo Tumor Growth Inhibition. We tested the effects of compound **24** on tumor growth by using a typical transplanted model applied to a syngenic hepatocellular carcinoma in Balb/c mice. Indeed, **24** showed significant in vitro cytotoxic activity (IC_{50} 3 μM) against BNL 1ME A.7R.1, a stabilized murine hepatocarcinoma cell line which is comparable to other hormone-responsive cell lines. As shown in Figure 7A, in mice implanted subcutaneously with 10^7 BNL 1ME A.7R.1 cells, a daily intraperitoneal injection of **24** (50 mg/kg) caused significant inhibition of tumor growth (65%) with respect to the control groups. Any tumor growth differences were not noted in those mice, which were treated both with phosphate-buffered saline and PEG suspension (vehicle). Daily administration of **24** (50 mg/kg), started 5 days after tumor implant, that is when the tumor mass had already been developed, caused 40% growth inhibition with respect to those mice which were given only vehicle (Figure 7B).

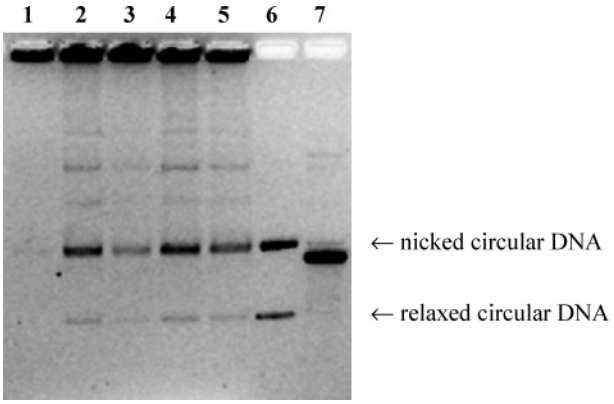


Figure 6. Antitopoisomerase II activity. Topoisomerase II decatenation assay in the presence of tested compounds: lane 1, KDNA control (no enzyme); lane 2, KDNA and topoisomerase II; lanes 3−5, same as lane 2 but with 0.8 μM **20**, **21** and **24**, respectively; lane 6, decatenated KDNA standard; lane 7, linear KDNA standard.

As we did not note any death, weight changes and histological abnormalities in liver and kidneys of the treated hosts, **24** appeared to be nontoxic under our experimental conditions. According to these results, the selectivity of the antimitotic effect, by means of which the 2-phenylpyrroloquinolin-4-ones **20**, **21** and **24** exerted their cytotoxic activity on the tested cell lines, may be attributed to their ability to inhibit the aromatase. This statement is also supported by recent binding models proposed for aromatase inhibitors, whether steroidal or not, and flavonoid compounds, illustrating some similarities on a molecular bases.[45,46] Some of these structural elements are also present in 2-phenylpyrroloquinolinones: the 4-oxo group ensures the interaction of the molecule with the heme prosthetic group of aromatase, and the pyrrole NH group simulates the 7-hydroxy group on the A ring of flavonoids, considerably enhancing their inhibitory potency. As flavones, substitutions on the 2-phenyl ring generally decrease the aromatase inhibitory potency of pyrroloquinolinone **24** (*m*-OCH_3) in comparison with **20** (Figure 5). The specific angular geometry of pyrrolo[2,3-*h*]quinolinone is the same as that 7,8-benzoflavone, which is a powerful inhibitor of aromatase.

Last, our findings on 2-phenylpyrroloquinolinones are consistent with a number of SARs and QSARs which have appeared during the past decade which reveal higher aromatase inhibitory activity for natural and synthetic flavones and flavanones carrying a phenyl ring in position 2, than for isoflavones and isoflavanones (phenyl in position 3), which are known to bind to estrogen receptors.[6,16]

Conclusions

When tested in vitro on a panel of 14 human tumor cell lines, some 2-phenylpyrroloquinolin-4-ones showed high and significant selective cytotoxic effect on hormone-sensitive cell lines. In view of the in vitro results and in vivo considerable growth inhibition of the hormone-sensitive BNL 1ME A.7R.1 tumor cell line implanted in Balb/c mice, these new compounds open new perspectives in the field of selective anticancer agents. Today, we may state that they belong to the antimitotic cancer drugs, as confirmed by flow cytometry and immuno-

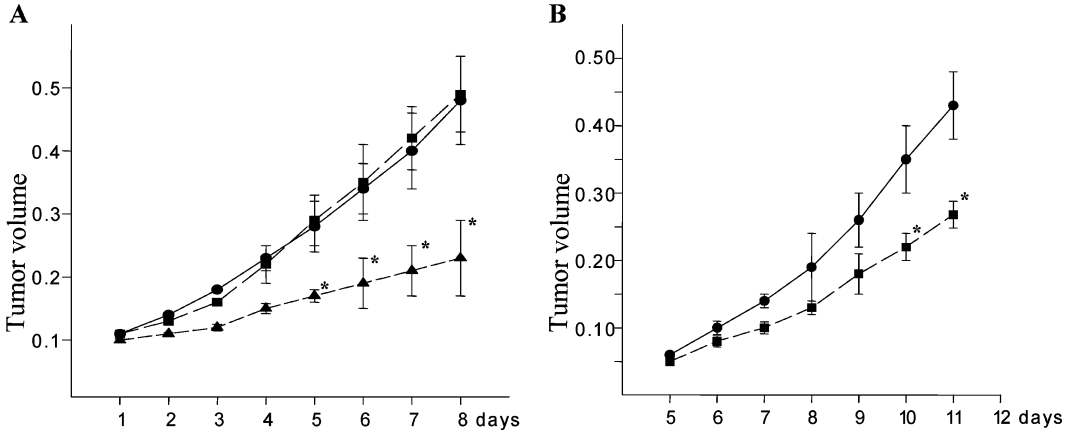


Figure 7. Antitumor activity of compound **24** observed in Balb/c mice carrying syngenic hepatocarcinoma. Male mice were injected subcutaneously with 10^7 BNL 1ME A.7R.1 cells, a syngenic hepatocellular carcinoma cell line, at their dorsal region. Figure 7A: starting on the second day, groups of animals (n = 8−12 per group) were administered daily ip with saline (circles), vehicle (squares) or 50 mg/kg body weight of **24** (triangles). Tumor size was measured daily over 10 days using calipers, and tumor volume (V) was calculated using the rotational ellipsoid formula: $V = A \times B2/2$, where A is the longer diameter and B the shorter diameter. Figure 7B: administration of vehicle (circles) or 50 mg/kg body weight of **24** (squares) was initiated 5 days after injection with tumor cells, and this treatment was continued for 7 days. Tumor size was measured daily as described above. Results are expressed as means ± SE. * indicates $P < 0.01$ vs vehicle or untreated animals.

fluorescence microscopy analysis, but unlike their parent 2-phenylquinolinones, they are selective for estrogen-sensitive cell lines, due to their aromatase inhibitory activity, as confirmed by the tritiated water release assay. Among the most active compounds **20**, **21** and **24**, only the unsubstituted 2-phenylpyrroloquinolinone **20** showed significant inhibitory activity of topoisomerase II.

All these results prompt us to design and synthesize more new pyrroloquinolinones in order to develop pharmacophoric elements directed toward mutual antimitotic action and aromatase inhibition with the ultimate aim of discovering new, more active, and more selective anticancer compounds.

Experimental Section

A. Chemistry. Melting points were determined on a Gallenkamp MFB 595 010M/B capillary melting point apparatus and are not corrected. Infrared spectra were recorded on a Perkin-Elmer 1760 FTIR spectrometer using potassium bromide pressed disks; all values are expressed in cm^{-1}. UV−vis spectra were recorded on a Perkin-Elmer Lambda UV/vis spectrometer. ^1H NMR spectra were recorded on Varian Gemini (200 MHz) and Bruker (300 MHz) spectrometers, using the indicated solvents; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. Coupling costants are given in hertz. In the case of multiplets, the chemical shift was measured 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, using a Perkin-Elmer 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 (70Ev) mass spectrometer and Applied Biosystems Mariner System 5220 LC/Ms (nozzle potential 250.00). Column flash chromatography was performed on Merck silica gels (250−400 mesh ASTM); chemical reactions were monitored by analytical thin-layer chromatography (TLC) using Merck silica gel 60 F-254 glass plates with a 9:1 dichloromethane/methanol mixture as eluant, unless otherwise specified.

Solutions were concentrated in a rotary evaporator under reduced pressure. Starting materials were purchased from Aldrich Chimica, Acros and Riedel-de Haen ($TiCl_3$, 15% HCl solution), and solvents from Carlo Erba, Fluka and Lab-Scan. Anhydrous DMSO was obtained by distillation under vacuum and stored on molecular sieves.

4-Aminoindole[28] **(1).** In a 50 mL round-bottomed flask, a solution of 2,6-dinitrotoluene (4.630 g, 25.4 mmol) and *N*,*N*-dimethylformamide dimethylacetal (6.8 mL, d = 0.89, 50.8 mmol) in anhydrous DMF (15 mL) was refluxed for 4 h, after which the starting material had completely disappeared, as shown by TLC analysis, leaving a spot at R_f = 0.25 (dichloromethane). The mixture was then evaporated to dryness and the resulting reddish-black oil treated with a 1:1 CH_2Cl_2/*n*-hexane mixture. After standing overnight at 4 °C, a red crystalline solid had formed, which was collected by filtration, washed with aqueous methyl alcohol and dried under vacuum. This turned out to be 2,6-dinitro-*trans*-β-dimethylaminostyrene, obtained in quantitative yields, and was directly used in the next reduction reaction ($TiCl_3$, 15% HCl solution) to give 4-aminoindole **1**.

4-Nitroindole[30,31] **(2).** In a one-necked flask equipped with a Claisen condenser were placed 2-methyl-3-nitro-aniline (6.00 g, 39.4 mmol), *p*-toluensulfonic acid (0.032 g, 0.2 mmol) and anhydrous triethyl orthoformate (9.1 mL, d = 0.891, 54.7 mmol); the mixture was heated at 120 °C until all the ethanol had distilled off. Then, following vacuum distillation at 150 °C and 0.6 mmHg, the imidate ester (6.5 g, 79.23%) was collected.

Without further purification, the imidate ester was used in the next reaction: in a 100 mL flask, 3.0 g (14.4 mmol) of imidate ester in 11.0 mL of anhydrous DMSO was treated with 3.0 mL of diethyl oxalate (21.7 mmol, d =1.076) in 7 mL of anhydrous DMF and 1.58 g (18.8 mmol) of potassium ethoxide at 0 °C. After 24 h at 40 °C, the reaction mixture was poured onto ice/water and the nitroindole separated as a brown crystalline product, subsequently purified by flash chromatography (CH_2Cl_2). R_f 0.55 (TLC, CH_2Cl_2).

***N*[1]-Methyl-4-nitroindole**[32] **(3).** To a stirring solution of 1 g (6.2 mmol) of 4-nitroindole in 30 mL of acetone were added 1.728 g of powdered KOH at 0 °C, giving a dark-red color, and then 0.77 mL (12.3 mmol) of CH_3I. After 2 h stirring at room temperature, the starting material had disappeared and a new spot formed at R_f = 0.51 (TLC, benzene/*n*-hexane/ethyl acetate 1:1:0.3). Following the addition of 180 mL of benzene to the reaction mixture, a precipitate formed, which was then eliminated by filtration. The filtrate was washed first with a NaCl saturated solution and then with water, dried over Na_2SO_4, and evaporated to dryness. The yellow residue was purified by flash chromatography, yielding 0.930 g (85.14%).

***N*[1]-Methyl-4-aminoindole**[28] **(4).** In a 150 mL flask, 0.396 g (2.2 mmol) of **3** was dissolved in 40 mL of acetic acid/water (2:1 mixture) by heating; to this orange solution were then added 11.56 mL of $TiCl_3$ 15% in HCl 5−10% (13.5 mmol) (Riedel-de Haën) at room temperature. The resulting brown mixture was stirred at the same temperature for about 2 h, the reaction being monitored by TLC (CH_2Cl_2/ethyl acetate 8:2, R_f 0.65). Last, water was added to the light yellow mixture, and a first extraction was made (CH_2Cl_2/methanol 9:1) to remove the starting product; the aqueous phase was made alkaline by concentrated NaOH and, following the addition of 30% NH_3, a second extraction was made with the same solvent mixture, to retrieve the amino derivative. The organic phase was washed with a saturated NaCl solution and H_2O and then dried with anhydrous Na_2SO_4. After solvent evaporation, 0.279 g of a brownish-red oil appeared which was purified by flash chromatography (CH_2Cl_2/ethyl acetate 8:2), yielding 0.195 g of a pure light yellow oil (60.6%).

2-[(1*H*-Indol-4-ylamino)methylene]malonic Acid Diethyl Ester (5). In a 50 mL round-bottomed flask were placed 0.265 g (2.00 mmol) of 4-aminoindole **1** and 0.405 mL (2.00 mmol, $d = 1.07$) of diethyl ethoxymethylenemalonate, and the mixtrue was heated at 130 °C for about 3 h. Any unreacted diethyl ethoxymethylenemalonate was removed under vacuum, leaving 0.500 g of a brown solid product (yield 83%); mp 122 °C (ethanol); $R_f = 0.83$ (CH_2Cl_2/ethyl acetate).

4-Oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*h*]quinolin-3-carboxylic Acid Ethyl Ester (6). A 0.500 g (1.7 mmol) amount of **5** was added to 50 mL of boiling diphenyl ether portionwise; after 20 min refluxing, the precipitate formed by cooling was collected by filtration, washed many times with diethyl ether and recrystallized from ethanol, yielding 0.360 g of pure tricyclic product (yield 83%); mp 280 °C (dec); $R_f = 0.45$ (ethyl acetate/methanol, 8:2).

4-Oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*h*]quinolin-3-carboxylic Acid (7). In a 100 mL round-bottomed flask were placed 0.260 g (1.01 mmol) of **6** and 30 mL of 2 N NaOH; the suspension was heated to complete solution and was then refluxed for 6 h, the reaction being monitored by TLC analysis (ethyl acetate/methanol, 8:2). After cooling, the reaction mixture was acidified to pH 5 by acetic acid/water 1:2. A brown precipitate formed, which was collected, washed with water and dried at 60 °C, yielding 0.185 g of a crystalline solid (yield 80%); mp 320 °C (dec); $R_f = 0.71$ (ethyl acetate/methanol, 8:2).

1*H*,7*H*-Pyrrolo[2,3-*h*]quinolin-4-one (8). In a two-necked 100 mL round-bottomed flask, 50 mL of diphenyl ether were heated to boiling temperature, and, following portionwise addition of acid **7** (0.185 g−0.81 mmol), the solution was refluxed for 20 min. After cooling, the formed precipitate was collected, washed many times with diethyl ether and dried, providing 0.120 g of a solid product (yield 80%); mp 351 °C (dec); $R_f = 0.38$ (ethyl acetate/methanol, 8:2).

3-Oxo-3-thiophen-2-yl-propionic Acid Ethyl Ester[20] **(9).** A 250 mL two-necked, round-bottomed flask equipped with a magnetic stirrer was fitted with a 100 mL pressure-equalizing constant-rate dropping funnel and a condenser, the top of which was connected to a mineral oil trap, to prevent air from entering the vessel during reaction and to monitor gas development. In the flask were subsequently placed 7.131 g (178.3 mmol) of sodium hydride (60% in mineral oil), which was then washed four times with 20 mL portions of benzene. Following the addition of 100 mL of fresh benzene and then 14.04 g (14.4 mL, $d = 0.975$, 118.9 mmol) of anhydrous diethyl carbonate, the mixture was heated under stirring. When it reached the boiling point, a solution of 2-acetylthiophene (6.42 mL, $d = 1.168$, 59.4 mmol) in benzene was added dropwise, slowly (1 h). When this addition was complete, the reaction mixture was refluxed until all hydrogen gas formation stopped. On cooling, a pasty, pink solid product separated; this was dissolved by adding 15 mL of glacial acetic acid dropwise, followed by 45 mL of ice-cold water. Once the organic layer was separated, the aqueous phase was extracted with benzene, and the organic extract washed with cold water, dried with anhydrous Na_2SO_4 and evaporated. Distillation under vacuum was needed in order to remove completely the unreacted diethyl carbonate and retrieve the desired product (130 °C, 0.6 mmHg) as a dense light orange oil (10.442 g, yield 89%); $R_f = 0.38$ (*n*-hexane/ethyl acetate 8:2).

3-(3-Methoxy-phenyl)-3-oxopropionic Acid Ethyl Ester[36] **(10).** This compound was prepared following the same method used for compound **9**, as reported in the literature.[37]

General Procedure for Synthesis of 3-(1*H*-Indol-4-ylamino)-3-substitutedacrylic Acid Ethyl Esters (11−17). In a 50 mL round-bottomed flask, 3−4 mmol of aminoindole **1** or **4** in 10−20 mL of absolute ethanol were condensed with an equimolar quantity of β-keto-ester (commercial **a**−**d** or prepared **11**, **12**) in the presence of 1 mL of glacial acetic acid and 100 mg of drierite. The mixture was refluxed for about 24 h, the reaction being monitored by TLC analysis (dichloromethane/ethyl acetate 9:1). 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 purified by flash chromatography and recrystallization from a suitable solvent.

3-Methyl-3-(*1H*-indol-4-ylamino)acrylic Acid Ethyl Ester (11). Brown oil, yield 51% (flash chromatography); $R_f =$ 0.86 (dichloromethane/ethyl acetate 9:1).

3-Thienyl-3-(1*H*-indol-4-ylamino)acrylic Acid Ethyl Ester (12). Yellow solid product purified by flash chromatography, yield 45.35%; mp 130−133 °C; $R_f = 0.92$ (dichloromethane/ethyl acetate 9:1).

3-Phenyl-3-(1*H*-indol-4-ylamino)acrylic Acid Ethyl Ester (13). Brown crystals, yield 50.31%; mp 135−136 °C; $R_f =$ 0.9 (dichloromethane/ethyl acetate 9:1).

3-Phenyl-3-(1-methyl-indol-4-ylamino)acrylic Acid Ethyl Ester (14). Dark-red vitreous solid by flash chromatography, yield 53%; $R_f = 0.96$ (dichloromethane/ethyl acetate 8:2); mp 55−60 °C.

3-(*p*-Nitrophenyl)-3-(1*H*-indol-4-ylamino)acrylic Acid Ethyl Ester (15). Brownish-red amorphous solid by flash chromatography, yield 59%; mp 143−145 °C; $R_f = 0.88$ (dichloromethane/ethyl acetate 9:1).

3-(*m*-Nitrophenyl)-3-(1*H*-indol-4-ylamino)acrylic Acid Ethyl Ester (16). Orange-red crystals obtained by flash chromatography and recrystallization from methanol. Yield 47%; mp 133−134 °C; $R_f = 0.90$ (dichloromethane/ethyl acetate 9:1).

3-(*m*-Methoxyphenyl)-3-(1*H*-indol-4-ylamino)acrylic Acid Ethyl Ester (17). Vitreous dark-red solid by flash chromatography. Yield 43.10%; mp 50−60 °C; $R_f = 0.87$ (dichloromethane/ethyl acetate 9:1).

General Procedure for Synthesis of 2-Substituted-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one Derivatives (18−24). In a two-necked round-bottomed flask, 50 mL of diphenyl ether were heated to boiling temperature; 1−2 mmol of acrylates **11**−**17** were then added portionwise, and the mixture was refluxed for 30 min. After cooling to 60 °C, the resulting precipitate was collected by filtration and washed many times with diethyl ether. In all cases, the collected products were purified by flash chromatography (ethyl acetate/methanol 9:1).

2-Methyl-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (18). Light brown solid from methanol, yield 70%; mp 302 °C (dec); $R_f =$ 0.13 (ethyl acetate/methanol 8:2).

2-Thienyl-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (19). Light brown solid from methanol, yield 35%; mp 293−294 °C (dec); $R_f = 0.51$ (ethyl acetate/methanol 8:2).

2-Phenyl-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (20). Crystalline product from ethanol, yield 69%; mp 319 °C (dec); R_f 0.37 (ethyl acetate/methanol 8:2).

2-Phenyl-*N*[7]-methyl-1*H*-pyrrolo[2,3-*h*]quinolin-4-one (21). Light brown crystals, yield 37%; mp 319−320 °C; $R_f =$ 0.26 (ethyl acetate/methanol 8:2).

2-(*p*-Nitrophenyl)-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (22). Light brown crystals, yield 56%; mp >300 °C; $R_f =$ 0.70 (dichloromethane/methanol 8:2).

2-(*m*-Nitrophenyl)-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (23). Yellow solid product, yield 57%; mp >300 °C (dec); $R_f = 0.36$ (dichloromethane/methanol 8:2).

2-(*m*-Methoxyphenyl)-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (24). Light crystals, yield 78%; mp 298−299 °C (dec); R_f = 0.37 (dichloromethane/methanol 8:2).

2-(*m*-Aminophenyl)-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one (25). In a 250 mL flask containing a solution of 0.400 g (1.3 mmol) of compound **23** in 120 mL of acetic acid/water 2:1 solution was added 6.74 mL of $TiCl_3$ 15% in HCl 5−10% (7.9 mmol) (Riedel-de Haën); the resulting mixture was stirred at room temperature for 3 h (TLC analysis, ethyl-acetate/methanol 8:2). Water was then added, and the mixture made alkaline with concentrated NaOH, followed by 30% NH_3. After extraction with ethyl acetate, treatment with a saturated NaCl solution of the organic layer and drying with anhydrous Na_2SO_4, yielded of a material, subsequently purified by flash chromatography (ethyl acetate/methanol 8:2) to give 0.240 g of a light yellow crystalline product (yield 67%); mp 183−186 °C; R_f = 0.49 (ethyl acetate/methanol 8:2).

2-(*m*-Aminophenyl)-1*H*,7*H*-pyrrolo[2,3-*h*]quinolin-4-one Dihydrochloride (26). A solution obtained by dissolving amino derivative **25** in a minimum quantity of heated absolute ethanol was saturated with dry HCl gas. After cooling to 0 °C, the dihydrochloride compound separated as a crystalline solid. Yield 93%. anal. ($C_{17}H_{13}N_3Cl_2$): C, H, N, Cl.

B. Biology. Cell Lines and Culture Conditions. Human cell lines, thyroid carcinoma (anaplastic C8305 and ARO), colon carcinoma (HT-29), pancreas adenocarcinoma (Mia Paca 2 and PT45), hepatocellular carcinoma (Hep 3B, Hep G2, PLC/PRF/5), glioblastoma (A-172), ovary carcinoma (OVCAR-3, IGROV-1), breast cancer (MCF-7), adrenocortical carcinoma (SW13 and NCI−H295) and a mouse hepatocellular carcinoma cell line (BNL 1ME A.7R.1) were grown at 37 °C in a humidified incubator in the presence of 5% CO_2, except for SW13, which needed no CO_2 to grow.

C8305, A-172, Mia Paca 2, PT45, IGROV-1 and OVCAR-3 cell lines were cultured in RMPI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin G and 10 μg/mL streptomycin; NCI-H295 cells were grown in RPMI supplemented with 2% FBS, 100 U/mL penicillin G and 10 μg/mL streptomycin, 1% insulin, transferrin, selenium-A (Gibco); ARO, Hep 3B, Hep G2, PLC/PRF/5, MCF-7, HT-29 and BNL 1ME A.7R.1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin G and 10 μg/mL streptomycin, and SW13 was grown in Leibovitz medium containing 10% FBS, 100 U/mL penicillin G and 10 μg/mL streptomycin. All cell lines were purchased from American Type Culture Collection.

In Vitro Cytotoxicity Assay. The cytotoxic activity of all new compounds was determined using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay.[7] Briefly, cell lines were seeded at a density of 10^4 cells/well in 96-well microtiter plates (Costar). After 24 h, exponentially growing cells were exposed to the indicated compound at a final concentration ranging from 0.01 to 100 μM. After 72 h, cell survival was determined by the addition of an MTT solution (10 μL of 5 mg/mL MTT in phosphate saline buffer).

After 4 h, 100 μL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 18 h; optical absorbance was measured at 550 nm by a LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentual values with respect to untreated cells. The IC_{50} value, i.e., the concentration of each compound required to reduce absorbency by 50% versus control cells, was determined from replicates of 6−8 wells from at least two independent experiments.

Fluorescence-Activated Cell Sorting Analysis. Hep G2, MCF-7, SW13 and Ovcar-3 cells were cultured for 24−48 h in a drug-free medium or supplemented with compound **24** (1−5 μM), vincristine sulfate salt (1 μM) or methotrexate (1 μM). As previously described,[47] cells were harvested with a cell scraper, washed twice with PBS and fixed in 70% cold ethanol (30 min at −20 °C). Cells (10^6) were then washed once in citrate phosphate buffer (0.2 N Na_2HPO_4 and 0.1 M citric acid, 24:1), followed by PBS, and finally incubated in an RNAse solution (100 μg/mL in PBS). After 30 min at 37 °C, the cells were incubated in a propidium iodide solution (PI, Sigma, 100 μg/mL in PBS) at room temperature for a further 30 min. To determine the effects of compound **24** on cell cycle dynamics, DNA fluorescence was measured by flow cytometry, analyzing at least 15 000 events with Lysis II software (Becton, Dickinson) at 488/525 nm (excitation/emission wavelength).

All experiments were repeated 3−4 times, and DNA content analysis was carried out using both logarithmic and linear scales. Results were comparable, irrespective of the scale used, and are shown on a logarithmic scale.

Apoptosis Assay. The capacity of compound **24** to induce apoptosis in tumor cell lines was assessed by quantifying histone-associated DNA fragments in the cytoplasm. OVCAR-3 cells were plated in quadruplicates at a concentration of 10^4 cells/well on 96-well plates. Following 24 h culture, compound **24** was added (1−5 μM) and, after 48 h incubation, cytoplasmic histone-associated DNA fragments were quantified by a commercially available cell death detection ELISA assay (Roche).

Indirect Immunofluorescence Detection of Microtubule Perturbation. MCF-7 and Ovcar-3 cells (3×10^4) were seeded on sterile microscope coverslips. After 24 h, compounds **20**, **21**, **24** (5 μM) and vincristine (1 μM) were added to the culture medium, and cells were incubated for 18 h. As described previously,[48,49] cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature, washed three times with PBS, permeabilized in 0.2% Triton X-100 in PBS for 10 min at room temperature and placed in methanol at −20 °C for 30 min. They were then washed with PBS, incubated for 1 h at 37 °C with 2% bovine serum albumin (BSA) and subsequently with a mouse monoclonal anti-β-tubulin antibody (diluted 1:400) for 1 h at 37 °C. Slides were washed three times with PBS and incubated with a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rat anti-mouse IgG (diluted 1:200 in a 2% BSA solution in PBS) for a further 1 h at 37 °C. Slides were then washed repeatedly with PBS, mounted with mounting medium and analyzed by cofocal microscopy (SP-2, Leyca) under green light.

Aromatase Activity Assay. Aromatase activity in subconfluent H295R cells was measured by the tritiated water release assay, with 0.5 μM [1β-^{3}H(N)]-androst-4-ene-3,17-dione (25.3 Ci/mmol; DuPont NEN, Boston, MA) as substrate.[42] Briefly, H295R cells were seeded at 10^6 cells/well on six-well plates and, after 48 h, were cultured for 24 h in DMEM-F12 in the absence or presence of FSK (25 μM). One hour before addition of the substrate, various concentrations of compounds **20**, **21** and **24** and letrozole were added. After a further 2 h exposure at 37 °C, 750 μL of incubation media was mixed with 5 volumes of chloroform and vortexed for 60 s to extract unconverted substrate. The aqueous phase was increased to 1.5 mL with distilled water and centrifuged at 800*g* for 5 min. An aliquot of ^3H_2O (1 mL) was placed in tubes containing 1 mL of 5% charcoal and 0.5% dextran T-70, vortexed for 60 s, and centrifuged at 9000*g* for 30 min. After centrifugation, 1 mL of the ^3H_2O phase was quantified by counting in 5 mL of Picofluor 15 premixed cocktail in a liquid scintillation counter. The results were expressed as pmoles [^{3}H]H_2O released per hour and were normalized for mg protein (pmol/h/mg protein). Protein yield was determined by the Bradford method.[50]

Activity Assays on Topoisomerase I and II. Relaxation Assay. Topoisomerase I activity was assayed by monitoring the relaxation of supercoiled plasmid pBR322 DNA. Reactions were performed in 22.5 μL of a reaction mixture containing 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, 0.25 μg pBR322 DNA (Sigma Chemical Co.), a test compound as indicated and 2 U of human topoisomerase I (TopoGEN, Inc.). Reactions were incubated at 37 °C for 30 min and then stopped by the addition of 2.5 μL of a solution containing 5% SDS and 5 mg/mL proteinase K (Sigma Chemical Co.) prewarmed at 37 °C for 30 min. After this time, 5 μL of stop buffer (50% sucrose, 50 mM EDTA, 0.1% bromophenol blue, 0.01% SDS) was added. Samples were analyzed by electrophoresis on a 1% agarose

gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Gels were then stained with 1 μg/mL ethidium bromide and subsequently destained with distilled water.

Decatenation Assay. Topoisomerase II activity was measured by the ATP-dependent decatenation of kinetoplast DNA (KDNA). The assay was performed using the topoisomerase II kit (TopoGEN, Inc.) and human topoisomerase II (TopoGEN, Inc.). Briefly, 20 μL reaction volumes containing 0.15 μg of KDNA, a test compound as indicated and 1 U of topoisomerase II were incubated for 1 h at 37 °C. After this time, 4 μL of stop buffer and 50 μg/mL of proteinase K were added and incubated for further 30 min at 37 °C. Samples were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL in TAE buffer. Gels were then destained in distilled water for 30 min at room temperature.

In Vivo Effects of Compound 24 on Tumor Growth. The test was conducted with a syngenic hepatocellular carcinoma model (BNL 1ME A.7R.1) in Balb/c mice. Male mice, 8 weeks aged, were purchased from Charles River (Calco, Lecco, Italy), and tumors were induced by a subcutaneous injection at both sides of 200 μL sterile PBS containing 10^7 BNL 1ME A.7R.1 cells. Animals were randomly divided in three groups, and starting on the second day, they were daily dosed intraperitoneally (ip) with 500 μL of saline, vehicle and **24** (50 mg/kg body weight) suspended in 0.9% NaCl containing 5% poly(ethylene glycol) and 0.5% Tween 80. Ten days later, animals were sacrificed and the tumor size was measured.[51]

In another set of experiments, drug administration started 5 days after the injection of tumor cells and lasted 7 days. All animals were then sacrificed and their tumors measured and harvested for pathological examination. In these experiments, the tumor size was measured every other day in anesthetized animals.

To measure the perpendicular diameter of each mass, calipers were used. In particular, the tumor volume (V) was calculated by the rotational ellipsoid formula: $V = A \times B_2/2$, where A is the longer diameter (axial) and B is the shorter diameter (rotational). All experimental procedures were accomplished following guidelines recommended by the Institutional Animal Care and Use Committee of Padua University.

Pathology. Mouse tissue specimens from the in vivo experiments were fixed in buffered 4% formalin for 24 h, and paraffin was embedded following dehydration. Cut sections (5 μm thick) were stained with hematoxylin/eosin and subjected to routine histological examination. To evaluate inflammatory infiltrates, tissue necrosis and presence of apoptotic cells, a microscopical examination was also carried out.

Statistical Analysis. Results are reported as means ± standard error (M ± SE). Statistical analysis was performed by using one-way variance analysis or Student's t-test, as appropriate. A P value of less than 0.05 was considered statistically significant.

Supporting Information Available: Spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, HRMS) and elemental analyses of all target compounds **8**, **18**−**25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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