Synthesis and Biological Activity of 7-Phenyl-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-ones: A New Class of Antimitotic Agents Devoid of Aromatase Activity

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The newly synthesized 7-phenyl-3*H*-pyrrolo[3,2-*f*]quinolinones **16**−**26** and previously **27** and **28** were assayed for their in vitro antiproliferative activity on tumor cell lines, and the lead compound **16** in vivo on a singenic hepatocellular carcinoma in Balb/c mice. Results from FACS, immunofluorescence microscopy analysis, tubulin polymerization assay, and tritiated water release assay for the CYP19 activity confirmed the new compounds as potential anticancer agents acting by tubulin depolymerization, but devoid of aromatase activity unlike their geometric [2,3-*h*] isomers.

Introduction

In chemotherapy, the search for multiacting drugs has always been a very attractive strategy, to increase effectiveness and selectivity and to overcome the drawbacks of toxicity and resistance.[1] In principle, molecules exhibiting several pharmacological properties simultaneously should display structural features suitable for interacting with various biological targets and consequently producing specific pharmacological effects. In this case, effects should aim at inhibiting tumor growth. With this aim in mind, we recently designed and characterized some 2-phenylpyrrolo[2,3-*h*]quinolin-4-ones[2] IV (Figure 1), as tricyclic aza-analogues of flavones. Aza-flavones 2-phenyl-quinolin-4-ones I (PQ),[3−7] 2-phenyl-quinazolinones II (PQZ),[8−12] and phenylnaphthyridinones III (PN)[13−15] (Figure 1) have been reported to possess potent in vitro cytotoxic activity but devoid of any selectivity.[16] Some of our phenylpyrroloquinolin-4-ones[2] IV were confirmed as being quite cytotoxic, with significant selectivity against estrogen-dependent growth cell lines. Studies of the mechanism of action confirmed the ability of the new tricyclic compounds to interfere with microtubule dynamics but also to inhibit aromatase, a key enzyme involved in estrogen biosynthesis.[2] Aromatase inhibitors are well-established drugs for treatment of estrogen-dependent breast cancer.[17,18] The latter unexpected activity was speculated to be due to the presence of structural elements such as those present in flavones (side phenyl ring, carbonyl group, and 7-NH group)[19−21] and to their tricyclic structure, with angular geometry similar to that of 7,8-benzoflavones V, powerful aromatase inhibitors (Figure 1).[22,23]

Now, to confirm the specific angular geometry of 2-phenyl-[2,3-*h*]pyrroloquinolin-4-ones IV as an element determining the selectivity and power of their cytotoxic effects, we undertook the synthesis and study of a series of geometric isomers in which the pyrrole ring is fused in positions 5 and 6 instead of 7 and 8 of 2-phenyl-4-quinolinone (Figure 1).

This paper reports the synthesis of eleven compounds, characterized by the pyrrolo[3,2-*f*]quinolin-9-one nucleus VI bearing various substituents on phenyl ring in position 7 and/or on the tricycle. Final compounds were also assayed for their in vitro antiproliferative activity on human and murine tumor cell lines and in vivo on a singenic hepatocellular carcinoma in Balb/c mice. To elucidate the mechanism(s) of action, the effect on cytoskeleton antimitotic activity was studied by fluorescence activated cell sorting analysis (FACS), immunofluorescence microscopy analysis, and tubulin polymerization assay, and their effect on CYP19 (aromatase) activity was examined by the tritiated water release assay.

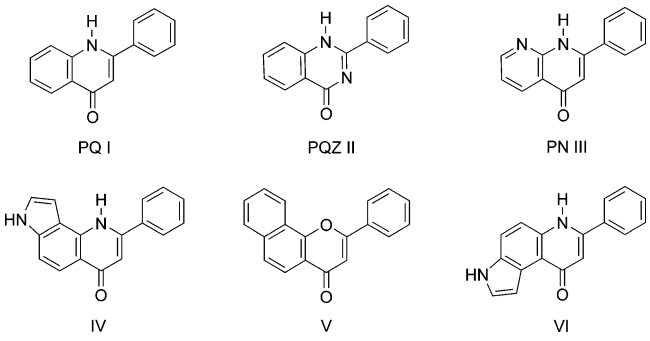

Figure 1.

Results and Discussion

Chemistry. In sections A and B of Scheme 1, the synthesis of ethyl substituted-benzoylacetates **1a**−**f** and 7-phenyl-pyrroloquinolinones **16**−**26** are reported following a method previously described[24] and recently adopted by us.[2] 5-Aminoindole derivatives **3**[26] and **4**,[27] obtained by reduction of the corresponding nitro compounds and the N^1-diethylaminoethyl-substituted **5** (from alkylation of **2**), were condensed with ethyl benzoylacetates **1a**−**f** and commercial ethyl 3-nitrobenzoyl- and benzoylacetates to give ethyl 3-indole-amino-3-phenylacrylates **6**−**15** in good yields. They were then cyclized to 7-phenyl-pyrroloquinolin-9-ones **16**−**26** in boiling diphenyl ether (250 °C). Amino derivative **23** was obtained by reduction with $TiCl_3$ reagent.

Biology. In Vitro Cytotoxic Activity. The in vitro cytotoxic activity of newly synthesized 7-phenyl-6,9-dihydro-pyrrolo[3,2-*f*]quinolin-9-ones **16**−**26**, 7-methyl-6,9-dihydro-pyrrolo[3,2-*f*]-quinolin-9-one **27**, and 6,9-dihydro-pyrrolo[3,2-*f*]quinolin-9-one **28** was studied on a panel of nine human and two murine tumor

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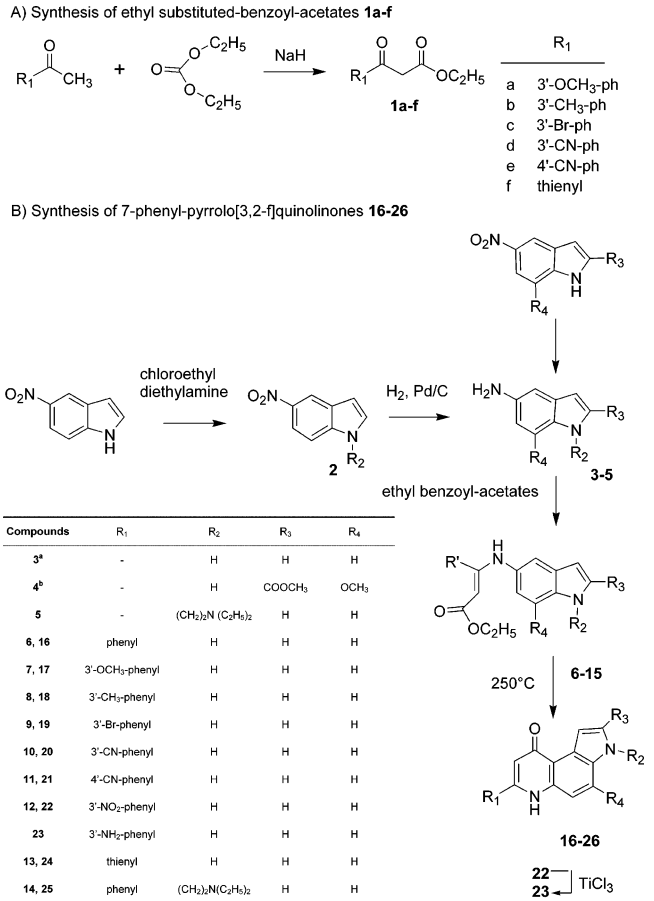
Scheme 1. Synthesis



Compounds	R_1	R_2	R_3	R_4
3[a]	-	H	H	H
4[b]	-	H	$COOCH_3$	OCH_3
5	-	$(CH_2)_2N(C_2H_5)_2$	H	H
6, 16	phenyl	H	H	H
7, 17	3'-OCH_3-phenyl	H	H	H
8, 18	3'-CH_3-phenyl	H	H	H
9, 19	3'-Br-phenyl	H	H	H
10, 20	3'-CN-phenyl	H	H	H
11, 21	4'-CN-phenyl	H	H	H
12, 22	3'-NO_2-phenyl	H	H	H
23	3'-NH_2-phenyl	H	H	H
13, 24	thienyl	H	H	H
14, 25	phenyl	$(CH_2)_2N(C_2H_5)_2$	H	H
15, 26	phenyl	H	$COOCH_3$	OCH_3
27[a]	CH_3	H	H	H
28[a]	H	H	H	H

a) ref. 26
b) ref. 27

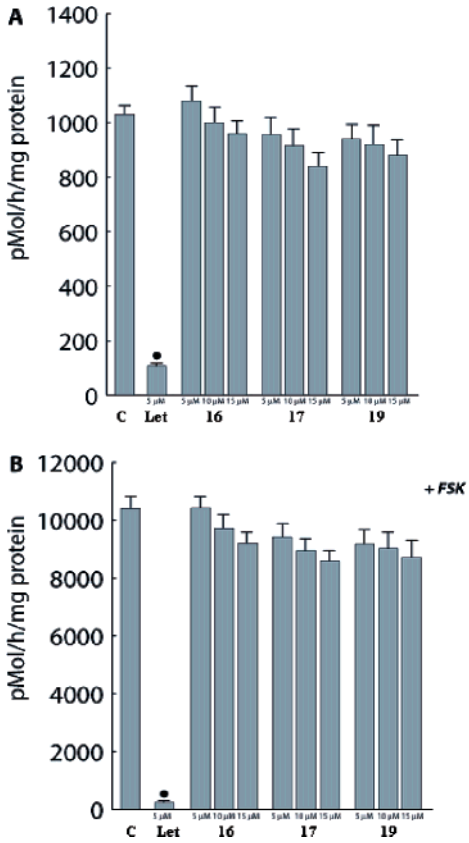


Figure 2. Effects of phenylpyrroloquinolinones **16**, **17**, and **19** on aromatase activity. H295R cells were cultured for 24 h in DMEM-F12 in absence (A) or presence (B) of FSK (25 μM). One hour before addition of substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione (0.5 μM), compounds **16**, **17**, **19**, and letrozole (Let) were added to cells at the specified concentrations. After a further 2-h exposure, aromatase activity was assessed using a modified tritiated water method. Results are expressed as pmol [^{3}H]H_2O released per hour and normalized for mg protein (pmol/h/mg protein). Values represent means ± SEM of three different experiments, each performed in triplicate.

cell lines by applying the MTT colorimetric assay.[28] Compounds were tested on a range of concentrations from 50 to 0.1 μM, and the calculated IC_{50} values are listed in Table 1. As shown, of all compounds tested, **16** and **17**, carrying a free phenyl ring or a 3′-methoxyphenyl group in position 7, were the most active, with IC_{50} values ranging from 0.4 to 8 μM. This is consistent with our previous data obtained with 2-phenyl-1,4-dihydropyrrolo[2,3-*h*]quinolin-4-ones[2] and with others previously reported for antitubulin PQ, PN, and PQZ.[3−16] Compounds **18**, **19**, **24**, and **25** showed cytotoxic activity with IC_{50} values ranging from 4 to 30 μM. 7-(Substituted-phenyl) **20**−**23**, 7-methyl **27**, and 7-unsubstituted **28**, carrying a phenyl ring with polar groups or lacking the phenyl ring, were only slightly or not at all active, with IC_{50} values generally higher than 30 μM. The chemically reactive cyano group was introduced at the 7-phenyl ring of pyrroloquinolinone (**20**, **21**), as it is also present in the most recent third-generation azole-type aromatase inhibitors,[29,30] but in this case it does decrease cytotoxic activity.

Table 1. In Vitro Cytotoxic Activity of 7-Substituted-6,9-dihydropyrrolo[3,2-*f*]quinolin-9-ones **16**−**28**

	cytotoxicity IC_{50} (μM)										
compd	Hela	Hep G2	H295R	Ovcar-3	MCF-7	Aro	A549	HT-29	PT-45	4T1	BNL
16	0.7	8	0.4	6	0.8	0.7	1	2	0.5	2	3
17	0.7	6	0.7	7	2	1	2	6	0.7	4	4
18	6	10	8	20	7	7	6	8	7	25	6
19	5	6	5	10	5	5	6	9	6	20	4
20	8	40	>50	>50	>50	3	>50	>50	40	>50	40
21	15	10	30	50	50	40	15	>50	20	25	15
22	25	30	40	>50	>50	39	32	>50	45	>50	>50
23	7	30	50	50	15	30	30	>50	8	50	>50
24	6	10	5	25	7	7	15	30	8	20	8
25	5	7	6	18	8	20	12	6	5	9	5
26	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
27[a]	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
28[a]	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
Taxol	0.008	0.3	0.08	0.01	0.007	0.0002	0.006	0.05	0.005	0.2	0.2
vincristine	0.005	0.2	0.09	0.02	0.02	0.0001	0.03	0.01	0.004	0.02	0.08

[a] Reference 26.

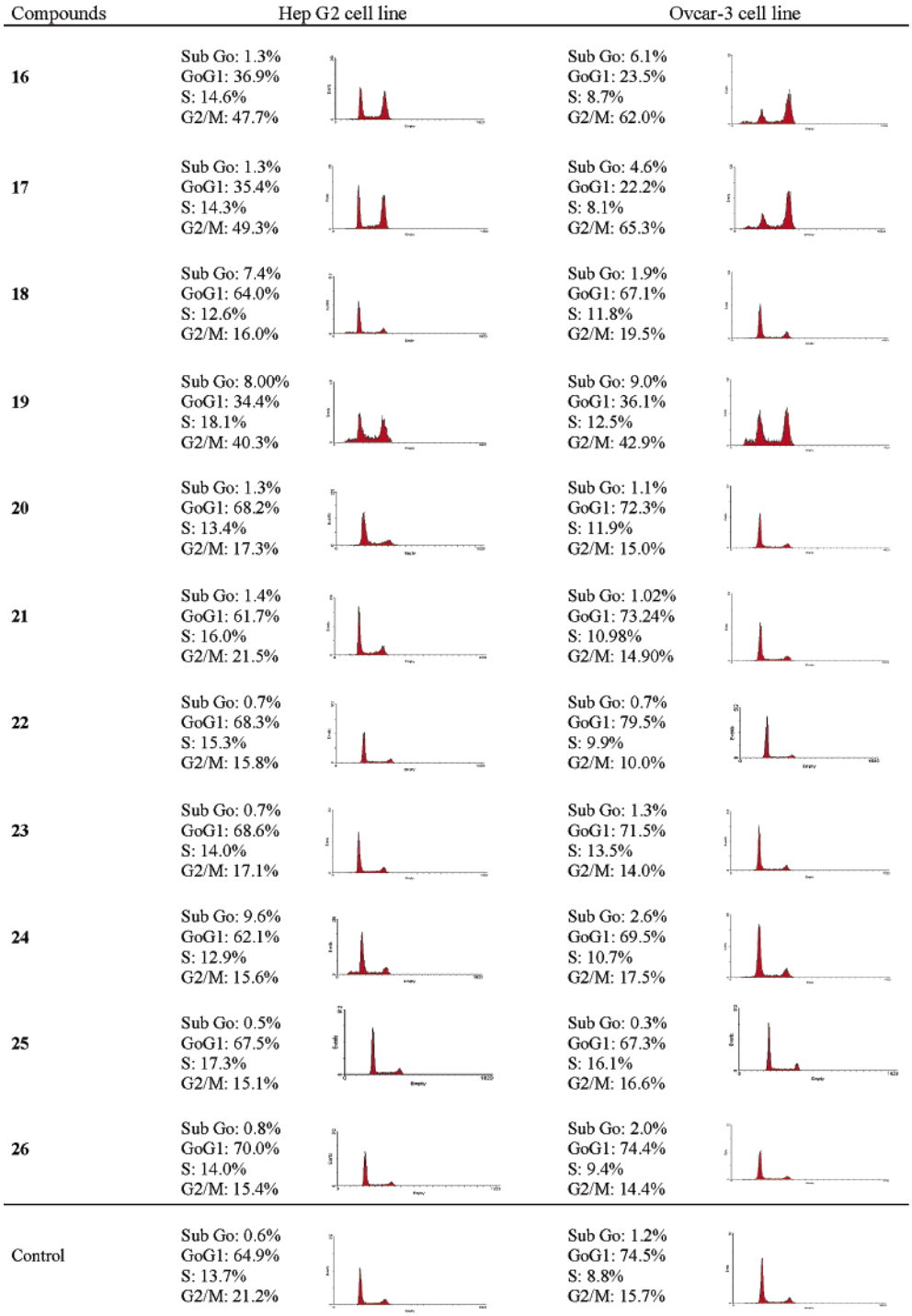


Figure 3. Flow-activating cell sorting analysis of phenylpyrroloquinolinones **16**−**26**. Effect of compounds **16**−**26** on cell cycle progression of Hep-G2 and Ovcar-3 cell lines was examined by staining cells with propidium iodide. Cells were treated with the different compounds at 10 μM (Hep G2) and 5 μM (Ovcar-3) and, after 24 h incubation, the percentage of cells in each phase was determined by FACS analysis and calculated using Lysis II software (Becton-Dickinson).

Compounds **22** (3′-NO_2-phenyl) and **23** (3′-NH_2-phenyl) were not cytotoxic. Thus, for this series of pyrroloquinolones, too, the presence of a phenyl ring is essential and, if unsubstituted, is preferable, due to its antiproliferative action.

It is interesting to note that compounds **25** ($5 < IC_{50} < 25$ μM), carrying the short diethylaminoethyl polar chain linked to the pyrrole N, and **26** ($IC_{50} > 50$ μM), with two oxygenated groups in positions 2 and 4, had lower activity in comparison with compounds **16** and **17** and to other analogues without polar chains at the pyrrole ring (data to be published). We therefore suggest an important structure−activity relationship regarding the polar nature of substitutions at the pyrrole ring of pyrroloquinolinones. Polar groups do not in fact promote cytotoxicity, perhaps by hindering or preventing useful interactions with lipophilic regions surrounding active tubulin sites.[31]

Comparing the new 7-phenylpyrrolo[3,2-*f*]quinolin-9-ones with the 2-phenylpyrrolo[2,3-*h*]quinolin-4-ones recently described by us (IC_{50} ranging from 8 to 0.7 μM),[2] we note that some of the former are more potent. Compounds **16** and **17** do show IC_{50} values under 1 μM for Hela, H295R, and PT-45 tumor cell lines (Table 1), but, looking at their cytotoxic profiles, they do not display any selectivity for estrogen-supported tumor

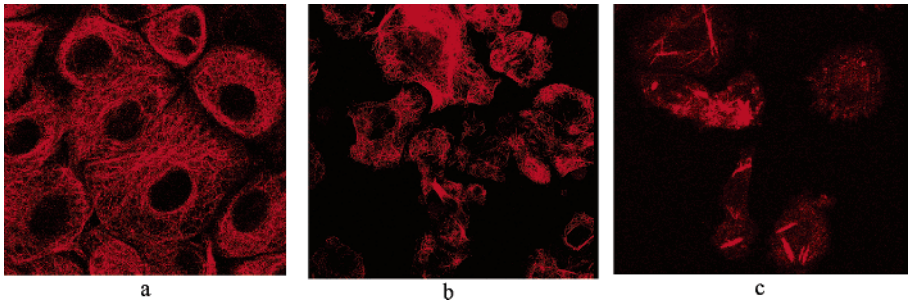


Figure 4. Immunofluorescence images of Ovcar-3 cells treated with **16**. Ovcar-3 cells were incubated for 18 h with medium drug-free (a), compounds **16** 5 μM (b), and vincristine 5 μM (c). Cells were then fixed and stained with anti-β-tubulin antibody, and microtubule distribution was visualized by fluorescence microscopy. Compound **16** completely destabilized microtubules in both interphase and mitotic cells, causing an abnormal microtubule network.

cell lines such as Ovcar-3, MCF-7, Hela, HepG2, and H295R, unlike their analogues. This suggests that the new compounds do not inhibit CYP-19 (aromatase) activity.

Aromatase Activity. The more cytotoxic compounds **16**, **17**, and **19** were subjected to the tritiated water release assay,[32] with letrozole as reference compound. At concentrations of 5−10−15 μM they did not show any inhibitory activity on aromatase. Taken together, the cytotoxicity properties (Table 1) and aromatase activity (Figure 2) of these compounds confirm that their specific geometry is the discriminating factor for possible selective effects in the pyrroloquinolinone family: derivatives with [2,3-*h*] geometry are antimitotics and aromatase inhibitors in estrogen-dependent tumor cell lines,[2] whereas derivatives with [3,2-*f*] geometry are only antimitotics (see below).

Flow-Activated Cell Sorting Analysis (FACS). To examine the effect of the new derivatives on cell cycle progression, flow activated cell sorting analysis (FACS) was performed. This method highlights the effects of drugs on the distribution of cells in specific phases: antimitotic drugs block cells in the G_2/M phase, causing an increment of the relative peak in the DNA histogram.[33,34] Eleven compounds, **16**−**26**, were tested on two cell lines (HepG2, Ovcar-3) at 5−10 μM. Figure 3 reports the results obtained after 24 h treatment for the active compounds **16**, **17**, and **19**. They present significant arrest of the cell cycle in G_2/M phase, raising the G_2/M peak from 21.2% (control) to 47.7% (**16**), 49.3% (**17**), and 40.3% (**19**) in Hep G2. In Ovcar-3, the same compounds increased the G_2/M peak from 15.7% (control) to 62.0% (**16**), 65.3% (**17**), and 42.9% (**19**). It is worthwhile to note that there is a good correlation between the cytotoxicity and the ability to block the cell cycle in G_2/M of these compounds, as **16**, **17**, and **19** turned out to be the most potent compounds in the MTT proliferation assay too (Table 1).

Immunofluorescence Microscopy. To prove that these new derivatives interfere with microtubule network, compound **16**, chosen as leader compound, was checked by immunofluorescence microscopy. As shown in Figure 4, compound **16** disrupted the tubulin network. Cells showed an evident characteristic "rounded up" morphology, caused by breaking up of microtubules in both interphase and mitotic cells after 18 h treatment at 5 μM, as for vincristine.

Effect on Tubulin Polymerization. We explored the effect of compound **16** on tubulin polymerization using a microtubule polymerization assay kit (Cytoskeleton), with taxol and vincristine as reference compounds. It is well-known that, in this assay, taxol causes a rise and vincristine a drop in the tubulin polymerization curve, as they respectively promote or prevent tubulin polymerization.[35,36] In Figure 5, compound **16** shows behavior similar to that of vincristine, displaying a good activity

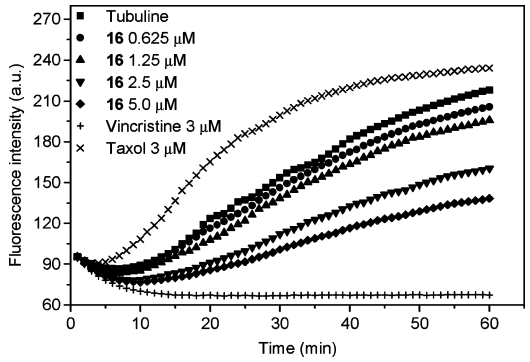


Figure 5. Tubulin polymerization assay. **"**In vitro" microtubule assembly of protein tubulin was followed fluorimetrically using a microtubule polymerization assay kit. Microtubule proteins were incubated with compound **16** at concentrations of 5, 2.5, 1.25, and 0.625 μM, and fluorescence (λ_{ex} 355 nm; λ_{em} 450 nm) was measured every minute for 60 min. Taxol and vincristine 3 μM were used as reference compounds.

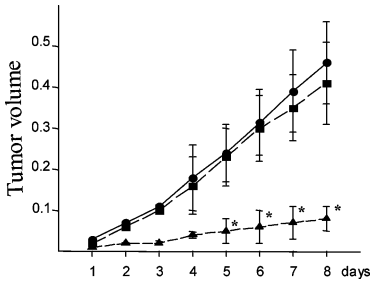


Figure 6. "In vivo" cytotoxic activity of compound **16**. Male mice were injected subcutaneously at their dorsal region with 10^7 BNL 1ME A.7R.1 cells, a syngenic hepatocellular carcinoma cell line. Starting on the second day, groups of animals (n = 8−12 per group) were administered daily ip with saline (circles), vehicle (squares), or 40 mg/kg body weight of **16** (triangles). Tumor size was measured daily over 10 days using calipers, and tumor volume (V) was calculated using the rotational ellipsoid formula: $V = A \times B^2/2$, where A is the longer diameter (axial) and B the shorter one (rotational). Results are expressed as means ± SE. ∗ indicates $P < 0.01$ vs vehicle or untreated animals.

at 5 and 2.5 μM, in a dose-dependent manner. We may therefore state that **16** exerts its antimitotic activity by tubulin depolymerization.

In Vivo Tumor Growth Inhibition. We tested the effects of compound **16** on tumor growth by using a typical transplanted model applied to a syngenic hepatocellular carcinoma in Balb/c mice because **16** showed significant in vitro cytotoxic activity (IC_{50} 3 μM) against BNL 1ME A.7R.1 cells. As shown in Figure 6, in mice implanted subcutaneously with 10^7 BNL 1ME A.7R.1 cells, a daily intraperitoneal injection of **16** (40 mg/Kg) caused a significant inhibition of tumor growth (83.2%) as compared

to mice receiving just vehicle. Further, any tumor growth difference was not noted in mice treated with PBS and PEG suspension. As we did not note any death, weight changes, and histological abnormalities in liver and kidneys of the treated hosts, **16** appeared to be nontoxic under our experimental conditions.

Conclusions

A series of new 3*H*-pyrrolo[3,2-*f*]quinolin-9-ones (**16**−**28**) was synthesized and studied as antiproliferative agents. 7-Phenyl derivatives **16**, **17**, and **19** showed considerable cytotoxic activity when subjected to the MTT assay on a panel of eleven human and murine tumor cell lines (IC_{50} ranging from 0.4 to 8 μM). To study their mechanism(s) of action, FACS analyses of compounds **16**−**26** were performed and showed that they provoke mitotic arrest. Immunofluorescence microscopy and tubulin polymerization assay of **16**, chosen as the lead compound, revealed relevant antimicrotubule effects by tubulin depolymerization. Compound **16** was tested "in vivo" in a syngenic model of murine hepatocarcinoma in Balb/c mice, showing remarkable antiproliferative activity, as it reduced the tumor volume by 83.2% within 10 days treatment with respect to the control group. In addition, compounds **16**, **17**, and **19** did not inhibit aromatase activity when subjected to the tritiated water release assay in H295R cells, thus suggesting that [3,2-*f*] pyrroloquinolinone geometry is not suitable for interfering with CYP19 aromatase, unlike the [2,3-*h*] geometry of recently described analogues. In conclusion, the mechanism of cytotoxicity of phenylpyrroloquinolinones described here refers only to antimitotic activity, as they share many features with compounds reported as microtubule destabilizing agents, such as the vinca alkaloids, vincristine and vinblastine.

Experimental Section

Chemistry. General Procedure for Synthesis of 6,9-Dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one Derivatives (16−**22, 24**−**26).** In a two-necked round-bottomed flask, 50 mL of diphenyl ether was heated to boiling temperature, 1−2 mmol of acrylates **6**−**15** was added portion-wise, and the mixture was refluxed for 30 min. After cooling to 60 °C, the separated 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).

7-Phenyl-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (16). Solid white product, yield 38%; mp >300 °C; R_f 0.67 (eluant ethyl acetate/methanol 9:1).

7-(3′-Methoxyphenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (17). Solid white product, yield 44%; mp 285−287 °C; R_f 0.69 (eluant ethyl acetate/methanol 9:1).

7-(3′-Methylphenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (18). Pale brown solid, yield 35%; mp >300 °C; R_f 0.62 (eluant ethyl acetate/methanol 9:1).

7-(3′-Bromophenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (19). Greenish-brown crystalline solid, yield 16%; mp 170−173 °C; R_f 0.53 (eluant ethyl acetate/methanol 9:1).

7-(3′-Cyanophenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (20). Pale brown solid, yield 58%; mp >300 °C; R_f 0.61; (eluant ethyl acetate/methanol 9:1).

7-(4′-Cyanophenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (21). Pale brown solid, yield 57%; mp >300 °C; R_f 0.66 (eluant ethyl acetate/methanol 9:1).

7-(3′-Nitrophenyl)-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (22). Yellow solid, yield 75%; mp >300 °C; R_f 0,54 (eluant ethyl acetate/methanol 9:1).

7-(3′-Amino)phenyl-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (23). Pale yellow, crystalline solid. Yield 45%; mp >300 °C; R_f 0.48 (ethyl acetate/methanol 9:1).

7-Thienyl-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (24). Pale brown solid, yield 59%; mp>300 °C; R_f 0.63 (eluant ethyl acetate/methanol 9:1).

7-Phenyl-3-(diethylaminoethyl)-6,9-dihydro-pyrrolo[3,2-*f*]-quinolin-9-one (25). Pale yellow solid, yield 59%; mp 85−90 °C; R_f 0.58 (eluant ethyl acetate/methanol 1:1).

2-Carboxymethyl-4-methoxy-7-phenyl-6,9-dihydro-3*H*-pyrrolo[3,2-*f*]quinolin-9-one (26). White crystalline solid, yield 35%; mp 321−323 °C; R_f 0.68 (eluant ethyl acetate/methanol 9:1).

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Supporting Information Available: Procedures for the synthesis of compounds **1a**−**f**, **2**, **5**, **6**−**15**, yields, chemical-physical properties (R_f, mp), and spectroscopic data of all synthesized compounds (IR, ^{1}H NMR, ^{13}C NMR), HRMS, the table of elemental analyses of all target compounds **16**−**26**, and the biological experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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