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Pyrrolo[3,2-*h*]quinazolines as Photochemotherapeutic Agents

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Heteroanalogues of angelicin, pyrrolo[3,2-*h*]quinazolines, were synthesized with the aim of obtaining new potent photochemotherapeutic agents. Many derivatives caused a significant decrease in cell proliferation in several human tumor cell lines after irradiation with UVA light (GI_{50} = 15.2–0.2 μM). Their phototoxicity effected apoptosis in Jurkat cells with the involvement of mitochondria (as determined by the loss of mitochondrial membrane potential and production of reactive oxygen species) and lysosomes. The phototoxicity of these compounds could be explained by lipid peroxidation.

Introduction

Angelicin (**1**), an angular furocoumarin, and its linear congener (psoralen) are photoactivable drugs which, owing to their planar structure, intercalate into DNA in the dark; upon photoactivation with UVA light, they generate covalent bonds with thymine bases. The linear shape of psoralen allows it to form both mono- and bis-adducts. In contrast, the angled geometry of angelicin restricts it to the formation of mono-adducts. The mono- and bis-adducts (ISC) thus formed are associated with short- and long-term side effects, respectively; the former include nausea, skin phototoxicity, and immune suppression, whereas the long-term risks consist of premature skin aging, collagen degeneration of dermal and elastic tissues, cataract induction, as well as mutagenicity and elevated risk of neoplastic disease.[1] Despite these undesirable side effects, furocoumarins are currently used in psoralen + UVA (PUVA) therapy for the treatment of several pathologies such as skin disorders (psoriasis, vitiligo), T-cell lymphomas, and T-cell-mediated autoimmune disease.[2] Much effort has gone into finding more active compounds with diminished side effects. Some particularly interesting improvements in PUVA therapy were made with the introduction of extracorporeal photopheresis (ECP), which was proposed in the mid-1980s by Edelson et al. for the treatment of T-cell skin lymphoma (CTCL), and which was approved by the US Food and Drug Administration (FDA) in 1988.[3] ECP represents a significant advance over conventional PUVA therapy, as patients are never directly exposed to UVA irradiation. Thanks to photopheresis, which consists of the reinfusion of extracorporeal UVA-irradiated autologous leukocytes incubated with methoxsalen (8-MOP), photochemotherapy with furocoumarins can be used for the treatment of tumors (i.e., lymphoma).[4] ECP is also used in the treatment of autoimmune diseases (systemic sclerosis and rheumatoid arthritis) as well as graft-versus-host disease (GVHD).[5]

Given our interest in pyrrole chemistry, we prepared heteroanalogues of angelicin. Among these, pyrano[2,3-*e*]isoindol-2-ones **2**[6a] and pyrrolo[3,4-*h*]quinolin-2-ones **3**[6b] (Figure 1) showed higher cytotoxicity than 8-MOP in some cases (2.7–17.8 μM and 0.2–15 μM, respectively). We recently reported the synthesis and biological activity of pyrrolo[3,4-*h*]quinazolines **4** (Figure 1).[7] Some of the synthesized compounds exhibited IC_{50} values in the micromolar range against leukemia and mela-

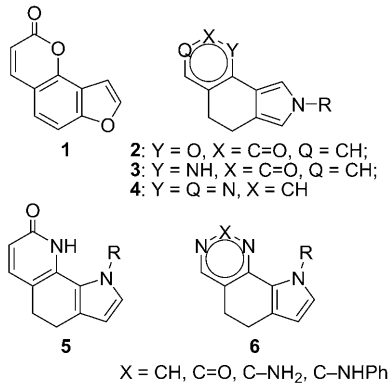

Figure 1. Structures of angelicin (**1**), pyrano[2,3-*e*]isoindol-2-ones **2**, pyrrolo[3,4-*h*]quinolin-2-ones **3**, pyrrolo[3,4-*h*]quinazolines **4**, pyrrolo[3,2-*h*]quinolinones **5**, and pyrrolo[3,2-*h*]quinazolines **6**.

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noma cell lines. We also investigated a different condensation of the pyrrole ring in the tricyclic system, leading to the pyrrolo[3,2-*h*]quinolinone **5**.[8] This compound class shows remarkable phototoxicity as photochemotherapeutic agents (0.5–7.2 μM), and studies designed to elucidate the mechanism of action revealed that they photo-induce cell death by apoptosis. However, the most important feature of these compounds is that they exert their remarkable activity without in vitro DNA damage, which is the main cause of the long-term side effects (mutagenesis and increased risk of cutaneous tumors) of PUVA therapy.

Encouraged by these results, we planned the annulation of the pyrimidine ring on the indole moiety in order to obtain derivatives of the pyrrolo[3,2-*h*]quinazoline (X = CH, C=O, C-NH_2, C-NHPh) ring system properly functionalized at position 2 of the pyrimidine moiety. While we were performing biological screens, similar derivatives were prepared as enzymatic inhibitors of Cdc7/Dbf4, showing micromolar activity "in the dark", but their photochemotherapeutic properties were not evaluated.[9]

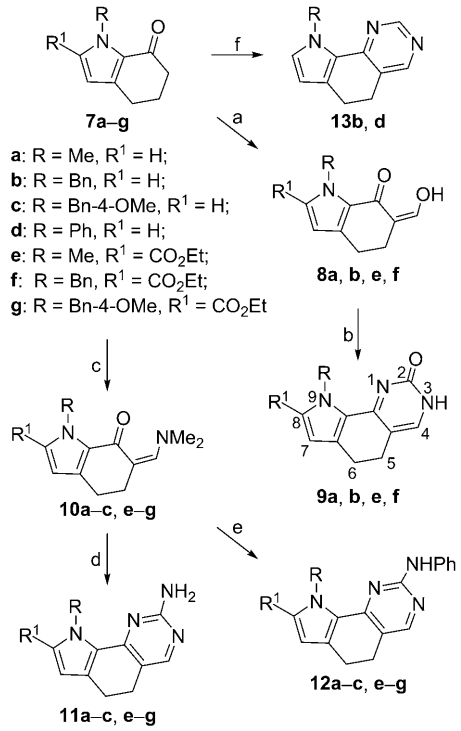
Herein we report the synthesis and evaluation of the photochemotherapeutic activity of the pyrrolo[3,2-*h*]quinazoline ring system **6**, which can considered a heteroanalogue of pyrroloquinolinones in which an additional nitrogen atom replaces C3 of the pyridone ring. The synthesis of this ring system was designed within the framework of heteroanalogues of angelicin, a model compound for photochemotherapy. However, in contrast to angelicin, our compounds, which are dihydro derivatives, are non-planar; we were nonetheless confident they would have good biological activity, as the four preceding series of angelicin heteroanalogues **2**–**5** are also dihydro derivatives and showed remarkable antiproliferative activity, with a mode of action different from that of the lead compound.

Results and Discussion

Chemistry

Among the various methodologies used for the synthesis of the pyrimidine framework, we focused our attention on the use of hydroxymethylene and enamino ketones as part of our project to investigate the reactivity of these synthons. In fact, it is well established that these key intermediates react with bidentate nucleophiles to furnish pyrans, pyrimidines, isoxazoles, and pyrazoles.[10] Tetrahydroisoindole-4-ones **7a**–**g**, suitable substrates for our purpose, were prepared by a method previously reported by us.[8] They are considered ideal building blocks in organic synthesis, as they can be easily functionalized with enamine or formyl functionalities at position α to the carbonyl group. The annular carbonyl group and the exocyclic carbon bound at position 6 are, in fact, two electrophilic centers readily available to react with dinucleophiles such as urea, guanidines, hydroxyamines, and cyanomethylenic compounds to achieve further annulation.[11]

In this light, ketones **7a**,**b**,**e**,**f** were treated, under a nitrogen atmosphere at 0 °C, with ethyl formate and potassium *tert*-butoxide in toluene to furnish hydroxymethylene ketones **8a**,**b**,**e**,**f** in good yields (65–80%; Scheme 1). Synthesis of pyrrolo[3,2-*h*]quinazolin-2-ones **9a**,**b**,**e**,**f** (60–68%) was readily accomplished by boiling the appropriate hydroxymethylene ketone in ethanol with urea. Interestingly, among the three possible



Scheme 1. *Reagents and conditions*: a) *t*BuOK, toluene, N_2, 0 °C, 3 h, then HCOOEt, 0 °C, overnight, 65–80%; b) urea, EtOH, reflux, 24 h, 60–68%; c) TBDMAM, DMF, MW (150 W, T = 190 °C), 5–15 min, 96–100%; d) $(NH_2)_2$–C=NH·HNO_3, EtOH, NaOMe, reflux, 2–3 h, 48–78%; e) $(NH_2)_2$–C=NHPh, DMF, reflux, 15 min–1.5 h, 46–70%; f) $CH(NHCHO)_3$, TsOH, formamide, N_2, reflux, 24 h, 45–54%.

tautomeric structures, compounds **9** exist in form A (Figure 2) as demonstrated by the presence of two doublets in the ^{1}H NMR spectra attributable to the exchangeable amidic proton at position 3 and to H-4 adjacent to it. This contrasts with the behavior of quinazolines, which generally exist in form C.[12] It is likely that steric hindrance of the substituent at position 9 (R group) causes the preference for form A.

In previous work, we prepared α-enamino ketones by treating tetrahydroindoles **7a**–**c**,**e**–**g** with the Bredereck reagent, *tert*-butoxybis(dimethylamino)methane (TBDMAM), to give compounds **10a**–**c**,**e**–**g** in excellent yields (80–90%).[7] Besides the conventional procedure, we further studied the reactivity

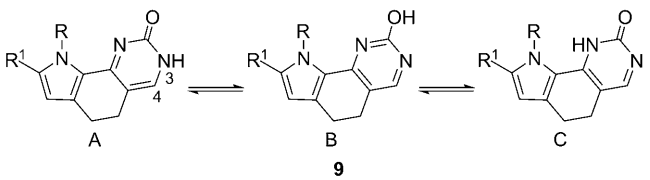


Figure 2. Tautomeric forms of compounds **9**.

of ketones **7** toward TBDMAM under microwave irradiation. Excellent results were obtained in terms of reaction times and yields by using *N*,*N*-dimethylformamide (DMF) as solvent and 1.5 equiv TBDMAM instead of the three equivalents necessary with the conventional method. In fact, under these conditions enamino ketones formed within 5–15 min, with a significant improvement in yields (96–100%); this made the workup procedures easier, as the crude materials, once poured onto crushed ice, precipitated to give the pure compounds.

The enamino ketones thus obtained were used as starting materials for the synthesis of 2-amino- and 2-anilino-pyrrolo-[3,2-*h*]quinazolines **11** and **12**, respectively. In particular, 2-amino compounds **11a**–**c**,**e**–**g** (48–78%) were obtained through reaction of enamino ketones with guanidine nitrate in the presence of sodium methoxide as the base to promote nucleophilic attack of the amino groups to the electrophilic centers, whereas 2-anilino derivatives **12a**–**c**,**e**–**g** (46–70%) were obtained by using phenylguanidine in DMF at reflux. Pyrrolo-[3,2-*h*]quinazolines **13** were obtained by the Bredereck method widely used for the synthesis of pyrimidines and their fused-ring derivatives, such as pyrimidinocarbazoles.[13] Thus, heating tetrahydroindole-4-ones **7b**,**d** at reflux in formamide with tris(-formylamino)methane in the presence of catalytic amounts of *p*-toluenesulfonic acid, derivatives **13b**,**d** were obtained in moderate yields (45–54%).

Biology

Antiproliferative activity

As pyrroloquinazolines were synthesized as antiproliferative compounds, their effect on cell survival was examined in a panel of human tumor cell lines: Jurkat (T-cell leukemia), K-562 (chronic myeloid leukemia) MCF-7 (breast adenocarcinoma), LoVo (colon adenocarcinoma), and A-431 (vulvar squamous cell carcinoma). The antiproliferative effects were evaluated by MTT assay after incubation of the cells with various concentrations of test compounds for 72 h; the results are listed in Table 1.[14]

Some compounds had no effect on cell proliferation at the concentrations used, and the share the same behavior as angelicin; others were active only in the Jurkat cell line (**9e** and **9f**). The compound that demonstrated a quite good antiproliferative activity is **12e**, with $GI_{50} < 5$ μM against both Jurkat and A-431 cell lines.

Evaluation of cell death mechanism

To evaluate the mechanisms of cell death induced by pyrroloquinazolines, some flow cytometry experiments were carried out with the compound that demonstrated the lowest GI_{50} value against the Jurkat cell line, **12e**. This study was performed by detection of the typical apoptotic loss of plasma membrane asymmetry or the characteristic rapid necrotic loss of plasma membrane integrity (Annexin V/propidium iodide (PI) test),[15] and through analysis of the cell-cycle profile of treated cells.[16]

Table 1. Antiproliferative activity of pyrrolo[3,2-*h*]quinazolines in a panel of tumor cell lines.

Compd	GI_{50} [μM]				
	Jurkat	K-562	MCF-7	LoVo	A-431
9a	>20[a]	>20	>20	>20	>20
9b	>20	>20	>20	>20	>20
9e	6.0±1.4	>20	>20	>20	>20
9f	8.4±1.2	>20	>20	>20	>20
11a	>20	>20	>20	>20	>20
11b	>20	>20	>20	>20	>20
11c	>20	>20	>20	>20	>20
11e	>20	>20	>20	4.2±0.8	>20
11f	14.0±1.3	>20	>20	14.2±0.2	>20
11g	>20	>20	>20	>20	>20
12a	12.0±1.5	>20	>20	14.7±0.5	9.3±1.2
12b	4.8±0.8	>20	>20	>20	5.0±0.4
12c	>20	>20	>20	>20	9.6±0.4
12e	2.9±0.5	13.1±1.1	>20	>20	1.4±0.1
12g	>20	>20	>20	>20	>20
13b	>20	>20	>20	>20	>20
13d	>20	>20	>20	>20	>20
Ang[a]	>20	>20	>20	>20	>20

[a] Angelicin.

In both tests, there was no evidence of cell death. In fact, in the first one, there was no increase in the percentage of cells showing exposure of phosphatidylserine (PS), a phospholipid normally found in the inner leaflet which is translocated to the outer leaflet during apoptosis; nor was there an increase in the number of cells with augmented permeability to PI, typical of necrosis, relative to control (Figure 3a). In the evaluation of cell-cycle profile (Figure 3b), no changes were detected for cells treated with **12e**, and in particular, there was no onset of the characteristic apoptotic peak, composed of cells with lower DNA content. The hypothesis is that pyrroloquinazolines are cytostatic but not cytotoxic compounds.

Phototoxicity experiments

As pyrroloquinazolines absorb in the UV range and have been demonstrated to undergo photolysis after UVA irradiation (see table 1 and figure 1 in the Supporting Information), their antiproliferative effect was examined after irradiation of a panel of human tumor cell lines in the presence of various concentrations of test compounds. These experiments were carried out in four cell lines: Jurkat, K-562, LoVo, and A-431. Cell survival experiments were carried out with two different UVA doses (2.5 and 3.75 $J\,cm^{-2}$, corresponding to 10 and 15 min irradiation time), using Wood's lamps emitting principally at $\lambda =$ 365 nm. For phototoxicity experiments, cells were incubated with compounds for 30 min prior to irradiation. Cell survival was monitored by MTT reduction assays 72 h after irradiation.[14]

Table 2 shows the extent of cell survival expressed as GI_{50}, which is defined as the concentration of test compound that elicits 50% inhibition of cell growth after irradiation at various UVA doses (2.5 and 3.75 $J\,cm^{-2}$). Almost all compounds presented UVA dose-dependent GI_{50} values in the low micromolar

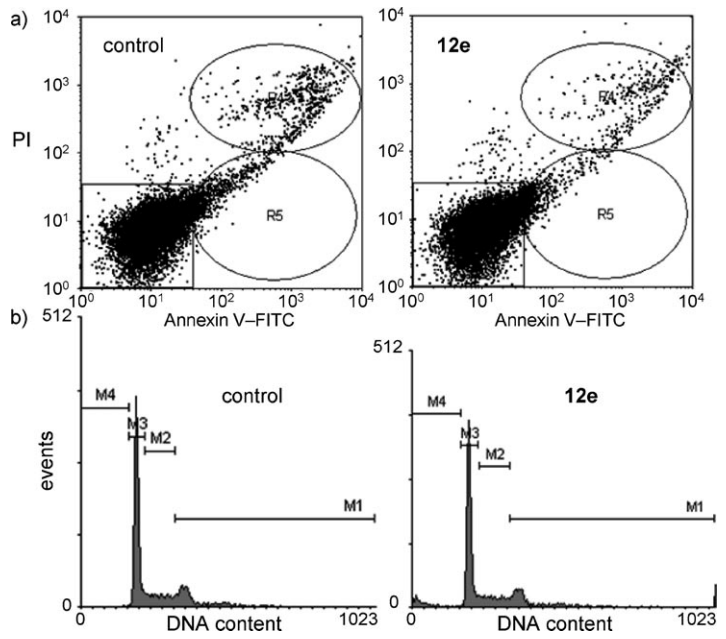


Figure 3. a) Exposure of PS and analysis of PI staining after treatment of Jurkat cells with 20 μM **12e** for 24 h. Cells were stained with Annexin V–FITC (FL1) and PI (FL3) and analyzed by flow cytometry; representative histograms are shown. b) Representative cell-cycle profiles of Jurkat cells incubated with 20 μM **12e** for 24 h. Cells were fixed and labeled with PI and analyzed by flow cytometry as described in the Experimental Section.

to sub-micromolar range; moreover, **11f** and **11g** consistently showed GI_{50} values <1 μM in all the cell lines tested.

Given the large number of compounds tested, some hypotheses can be formulated regarding the structure–activity relationships (SAR). Pyrrolo[3,2*h*]quinazolines were variably substituted at positions 2, 8, and 9. In all compounds, the nature of the substituent at position 2 appears to play an important role in the induction of phototoxicity; in fact, the antiproliferative activity generally decreases along the following ranking: amino group $>$ aniline group $>$ carbonyl group $\geqq$ no substitution. In general, the increase in steric hindrance of the substituent at position 9 (methyl $<$ benzyl $\approx$ *p*-methoxybenzyl) augmented phototoxicity, and the introduction of an ethoxycarbonyl moiety at position 8 also improved the phototoxicity, or even led to active compounds (for example: from the inactive compound **9a**, **9b**, and **11a** to the phototoxic analogues **9e**, **9f**, and **11e**). Moreover, the low phototoxic potential of some compounds (**12e**, **13b**, and **13d**) could be justified by the low ε value at $\lambda =$ 365 nm, as illustrated in table 1 of the Supporting Information.

Evaluation of cell death mechanisms

We performed some experiments (Annexin V/PI test) to analyze the mechanisms of cell death with the two most phototoxic compounds, **11f** and **11g**.[15] As illustrated in Figure 4, treated cells demonstrated time-dependent features of apoptotic cell death. After 24 h (Figure 4a), most of the cells were in the late-apoptotic stage (grey bars). Therefore, we decided to monitor cell death after a shorter incubation time. After 3 h from irradiation, an increase in early-stage apoptotic cells (black bars) and late-stage apoptosis was detected for both compounds in a concentration-dependent manner relative to control (Figure 4b). A small percentage of necrotic cells was also detected, but the primary mechanism of cell death seemed to be apoptosis.

Evaluation of mitochondria involvement in cell death

Mitochondria have been suggested as critical players in apoptosis, and many signals for mitochondrial involvement have

Table 2. Photocytotoxicity of pyrrolo[3,2-*h*]quinazolines in a panel of human cell lines.

Compd	GI_{50} [μM][a]							
	Jurkat		K-562		LoVo		A-431	
UVA [J cm^{-2}]:	*2.5*	*3.75*	*2.5*	*3.75*	*2.5*	*3.75*	*2.5*	*3.75*
9a	>20[b]	>20	>20	>20	>20	>20	>20	>20
9b	>20	>20	>20	13.69 ± 1.09	>20	>20	>20	>20
9e	4.85 ± 0.53	3.47 ± 0.11	5.70 ± 0.76	4.38 ± 0.44	9.72 ± 1.32	6.76 ± 0.57	>20	8.05 ± 1.69
9f	2.05 ± 0.62	1.08 ± 0.25	7.96 ± 1.46	2.92 ± 0.42	3.07 ± 0.35	2.30 ± 0.24	3.91 ± 0.15	2.45 ± 0.38
11a	>20	8.42 ± 1.49	>20	>20	>20	>20	>20	>20
11b	0.65 ± 0.13	0.39 ± 0.05	1.44 ± 0.12	0.72 ± 0.28	0.85 ± 0.19	0.54 ± 0.09	1.19 ± 0.03	0.96 ± 0.05
11c	0.43 ± 0.08	0.28 ± 0.08	1.71 ± 0.17	0.53 ± 0.09	1.12 ± 0.23	0.59 ± 0.18	1.31 ± 0.17	0.60 ± 0.16
11e	0.83 ± 0.16	0.46 ± 0.09	2.38 ± 0.70	1.16 ± 0.20	1.57 ± 0.12	1.02 ± 0.09	1.90 ± 0.11	1.34 ± 0.25
11f	0.43 ± 0.08	0.21 ± 0.06	0.74 ± 0.13	0.48 ± 0.08	0.56 ± 0.07	0.44 ± 0.04	0.74 ± 0.07	0.39 ± 0.10
11g	0.29 ± 0.02	0.23 ± 0.01	0.75 ± 0.18	0.41 ± 0.05	0.47 ± 0.07	0.34 ± 0.03	0.44 ± 0.05	0.26 ± 0.06
12a	0.83 ± 0.08	0.55 ± 0.09	3.51 ± 0.80	1.82 ± 0.39	0.82 ± 0.14	0.55 ± 0.08	2.28 ± 0.18	1.44 ± 0.15
12b	0.81 ± 0.07	0.52 ± 0.11	3.90 ± 0.70	2.85 ± 0.20	2.31 ± 0.17	0.95 ± 0.09	1.77 ± 0.27	1.16 ± 0.12
12c	2.05 ± 0.12	0.68 ± 0.09	4.06 ± 0.60	2.16 ± 0.28	2.80 ± 0.29	1.43 ± 0.15	3.79 ± 0.43	2.53 ± 0.53
12e	>20	8.53 ± 1.53	>20	>20	15.21 ± 1.97	9.61 ± 1.32	>20	5.69 ± 1.32
12g	0.70 ± 0.11	0.50 ± 0.05	2.63 ± 0.36	1.88 ± 0.35	0.88 ± 0.11	0.48 ± 0.11	1.57 ± 0.11	1.09 ± 0.21
13b	>20	>20	>20	>20	>20	>20	>20	>20
13d	>20	>20	>20	>20	>20	>20	>20	>20
Ang[b]	1.00 ± 0.20	0.90 ± 0.10	1.21 ± 0.11	1.09 ± 0.10	4.08 ± 0.41	1.12 ± 0.44	5.09 ± 0.65	2.15 ± 0.35

[a] Values represent the mean $\pm$SEM of at least three experiments. [b] Angelicin.

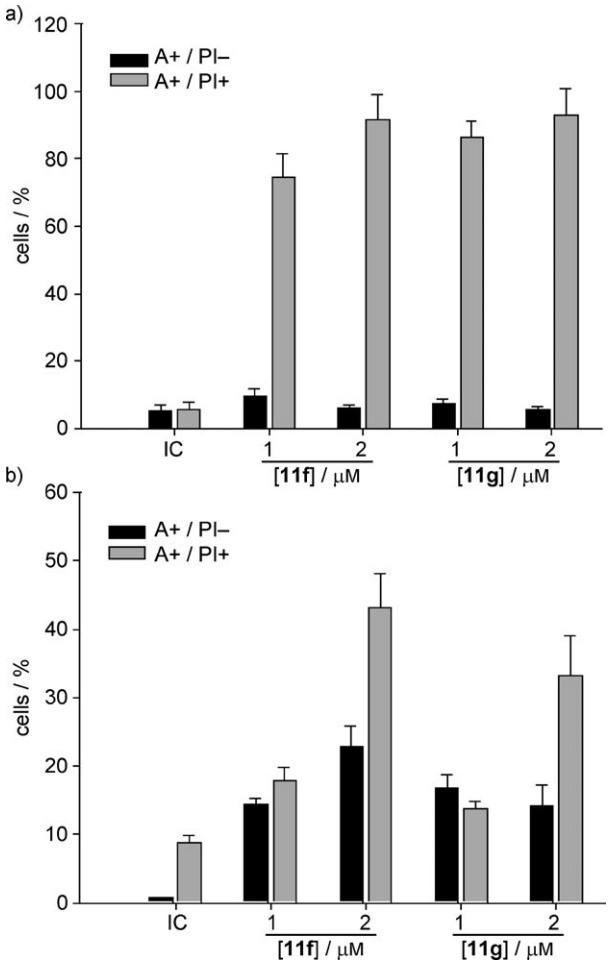


Figure 4. Exposure of PS and analysis of PI staining a) 24 and b) 3 h after irradiation (2.5 J cm^{-2}) of Jurkat cells treated with **11 f** or **11 g** at 1 or 2 μM. Cells were stained with Annexin V–FITC (FL1) and PI (FL3) and analyzed by flow cytometry; the percentage values represent the mean of three independent experiments. IC = irradiated control; A + /PI − = fluorescence in FL1 and no fluorescence in FL3; A + /PI + = fluorescence in FL1 and FL3.

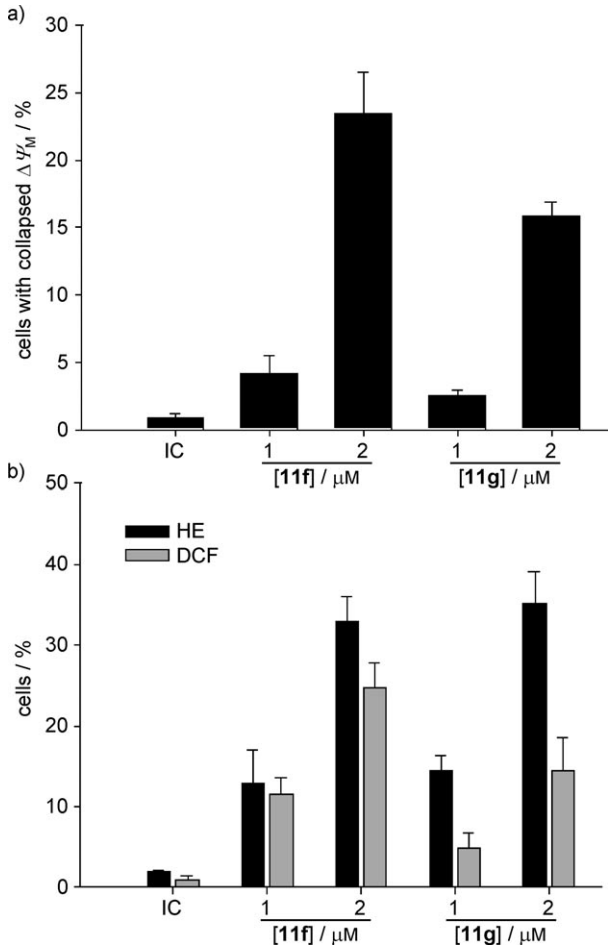


Figure 5. Mitochondrial involvement in photo-induced cell death. a) Percentage of cells with loss of mitochondrial membrane potential ($\Delta\Psi_M$) measured by JC-1 staining 3 h after irradiation (2.5 J cm^{-2}) of Jurkat cells treated with **11 f** or **11 g** at 1 or 2 μM; cells were stained with JC-1 and analyzed by fluorescence-activated cell sorting (FACS). b) Production of ROS in Jurkat cells 6 h after irradiation (2.5 J cm^{-2}) and treatment with **11 f** or **11 g** at 1 or 2 μM; cells were stained with HE or DCFDA and analyzed by FACS. Values represent the mean ± SEM of three independent experiments. IC = irradiated cells without compound.

been observed: release of apoptotic factors such as cytochrome *c* and apoptosis-inducing factor (AIF), translocation of some Bcl-2 family proteins to mitochondria such as tBid, and failure of mitochondrial functions such as loss of mitochondrial membrane potential.[17]

Mitochondrial involvement in apoptosis was determined by examining some features of this organelle's dysfunction, such as the loss of mitochondrial membrane potential ($\Delta\Psi_M$) and the production of reactive oxygen species (ROS). The $\Delta\Psi_M$ was monitored by fluorescence of the dye JC-1, which has the unique property of changing its fluorescence properties with the loss of mitochondrial potential. In fact, it forms orange fluorescent aggregates locally and spontaneously under high mitochondrial $\Delta\Psi_M$, whereas after loss of potential, JC-1 is present in monomeric form, which fluoresces in green.[18]

Irradiated Jurkat cells in the presence of compounds **11 f** and **11 g** exhibit a shift in fluorescence in a dose-dependent manner relative to control cells (Figure 5 a), indicating depolarization of mitochondrial membrane potential. The percent loss of mitochondrial potential is consistent with the percentage of early-stage apoptotic cells in the previous experiment.

ROS production, a consequence of mitochondrial electron chain disruption, was examined by flow cytometry 6 h after irradiation of Jurkat cells in the presence of **11 f** and **11 g** to exclude the influence of compound photochemical reactions. Cells were stained with hydroethidine (HE) and 2,7-dichlorodihydrofluorescein diacetate (DCFDA), which fluoresce upon oxidation with superoxide anion and hydrogen peroxide, respectively.[19] Irradiated Jurkat cells in the presence of compounds **11 f** and **11 g** presented a clear increase in fluorescence in a dose-dependent manner in comparison with control cells (Figure 5 b).

Evaluation of lysosome involvement in cell death

Lysosomal alterations can be involved in cell death caused by many photosensitizers such as certain fluoroquinolones,[20] acri-

dine orange,[21] and some psoralen analogues.[7] To investigate the integrity of lysosomes after irradiation in the presence of **11 f** and **11 g**, flow cytometric analysis was performed with the fluorescent dye acridine orange (AO). AO is a lysosomotropic base and a metachromatic fluorochrome with red fluorescence when highly concentrated in intact lysosomes and with green fluorescence at low concentrations, as in damaged lysosomes.[22] The percentage of cells with intact lysosomes can be evaluated by assaying red fluorescence after AO staining of cells exposed to the photosensitizer. A significant extent of lysosomal damage was photo-induced by **11 f** and **11 g** 3 h post-irradiation (Figure 6).

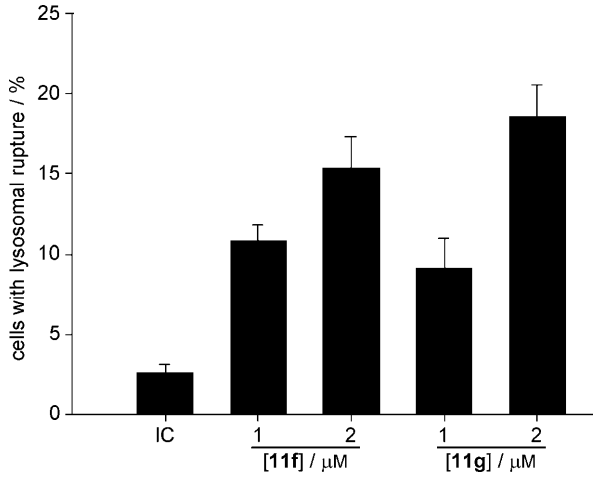


Figure 6. Lysosomal dysfunction after irradiation of Jurkat cells in the presence of pyrroloquinazolines. Percentages of cells stained with AO were analyzed by flow cytometry 3 h after irradiation (2.5 J cm^{-2}) in the presence of **11 f** or **11 g** at 1 or 2 μM. Values represent the mean ± SEM of three independent experiments. IC = irradiated cells without compound.

Lipid peroxidation

The thiobarbituric acid (TBA) assay was performed to determine potential lipid peroxidation 24 h post-irradiation of Jurkat cells in the presence of test compounds. Briefly, this assay involves the reaction of one molecule of malondialdehyde (MDA), which is a secondary product of lipid peroxidation, with two molecules of TBA. The formation of the reaction product, a pink chromogen, was monitored by fluorescence at $\lambda = 550$ nm.[23] A significant concentration-dependent production of thiobarbituric acid reactive substances (TBARS) was observed when cells were exposed to the test compounds and UVA (Figure 7).

Conclusions

In summary, we have described the synthesis and some photobiological properties of new angelicin heteroanalogues based on the pyrroloquinazoline scaffold with the aim of identifying new photochemotherapeutics. Some of these derivatives were found to inhibit cell proliferation in vitro by MTT assays, even in the absence of UVA irradiation. However, after flow cytometric analysis, cytotoxic activity was excluded, as no increase in cell death was detected in treated samples relative to control cells, whereas cytostatic activity can explain the decrease in cellular metabolic activity observed in MTT tests. Pyrrolo[3,2-*h*]quinazolines absorb in the UV and UVA range, and many of these derivatives undergo photolysis to varying degrees, as evidenced by monitoring changes in their structure after UVA irradiation.

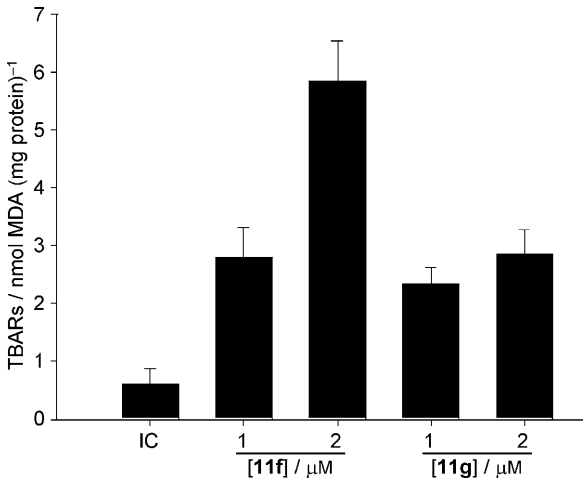


Figure 7. Lipid peroxidation induced in Jurkat cells 24 h after irradiation (2.5 J cm^{-2}) in the presence of **11 f** or **11 g** at 1 or 2 μM. The TBA test was performed on the supernatant medium. Values represent the ± SEM of three independent experiments. IC = irradiated cells without compound.

Most derivatives showed remarkable phototoxicity in many human tumor cell lines, reaching GI_{50} values in the low micromolar and sub-micromolar range. The phototoxic potency of the most active derivatives was higher not only than angelicin (**1**), but also the other pyrroloquinolinones **3** and **5** and pyranoisoindolone analogues **2**. The most phototoxic compounds, **11 f** and **11 g**, bear an amino group, a substituent of steric bulk, and an ethoxycarbonyl moiety at positions 2, 9, and 8, respectively.

Pyrrolo[3,2-*h*]quinazolines induced significant cell death at only 3 h after UVA irradiation, as detected by Annexin V/PI test. These derivatives elicit cell death by apoptosis as is the case with the psoralens.[24] We also carried out some experiments to determine which organelles play important roles in the cell-death process; for PUVA therapy, many reports have demonstrated the importance of mitochondria.[24,25] Two organelles, mitochondria and lysosomes, are heavily involved in inducing cell death, as we demonstrated a collapse in mitochondrial membrane potential, production of ROS, and the disruption of lysosomal integrity.

The lipid peroxidation induced by pyrrolo[3,2-*h*]quinazolines could explain their phototoxic activity. Although we have identified a very interesting class of new derivatives with high phototoxicity, further studies are required to identify the initial signal(s) and downstream events involved in the activation of apoptosis. Identification of these specific targets may have significant implications in the therapeutic application of these derivatives.

Experimental Section

Chemistry

General. All melting points were taken on a Büchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined in $CHBr_3$ with a Jasco FTIR 5300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz, in [D_6]DMSO or $CDCl_3$ solution, using a Bruker Avance II series 200 MHz spectrometer ($(CH_3)_4Si$ as internal reference). Column chromatography was performed with Merck silica gel 230–400 mesh ASTM or with a Büchi Sepacore chromatography module (pre-packed cartridge system). Elemental analyses (C, H, N), performed with a Vario EL III elemental analyzer, were within ± 0.4% of the theoretical values.

Compounds **7a**–**g** were prepared as previously described.[7]

General procedure for the synthesis of 6-(hydroxymethylene)-1,4,5,6-tetrahydro-7*H*-indole-7-ones (8a,b,e,f). To a suspension of *t*BuOK (1.03 g, 9.2 mmol) in anhydrous toluene (40 mL), under N_2 at 0 °C, a solution of the suitable ketone **7** (4.6 mmol) in the same solvent (15 mL) was added, and the reaction mixture was stirred for 3 h. A solution of ethyl formate (0.56 mL, $d = 0.921$ g mL^{-1}, 6.9 mmol) in anhydrous toluene (10 mL) was then added, and the reaction was stirred overnight. The solvent was removed in vacuo, and H_2O (50 mL) was added. The aqueous phase was extracted first in basic medium (NaOH, 4%) with EtOAc (5×100 mL) and the organic layer discarded. The aqueous layer was acidified (3 N HCl) and extracted; the organic layers were dried (Na_2SO_4), and removal of the solvent gave the crude which was purified by column chromatography eluting with CH_2Cl_2/EtOAc (99:1).

6-(Hydroxymethylene)-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (8a). This compound was obtained from **7a** as a brown oil (0.59 g, 72%): $R_f = 0.60$ (CH_2Cl_2/EtOAc, 95:5); ^1H NMR ($CDCl_3$): $\delta =$ 2.49 (t, $J = 6.5$ Hz, 2H, CH_2), 2.70 (t, $J = 6.5$ Hz, 2H, CH_2), 3.95 (s, 3H, CH_3), 5.98 (d, $J = 2.1$ Hz, 1H, H-3), 6.78 (d, $J = 2.1$ Hz, 1H, H-2), 7.19 (d, $J = 10.3$ Hz, 1H, CH), 13.63 ppm (d, $J = 10.3$ Hz, 1H, OH); ^{13}C NMR ($CDCl_3$): $\delta = 23.3$ (t), 26.1 (t), 36.4 (q), 107.0 (d), 109.7 (s), 126.9 (s), 131.3 (d), 136.6 (s), 159.9 (d), 183.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3391$ (OH), 1633 cm^{-1} (CO); Anal. calcd (%) for $C_{10}H_{11}NO_2$ (177.20): C 67.78, H 6.26, N 7.90, found: C 67.86, H 5.98, N 8.10.

1-Benzyl-6-(hydroxymethylene)-1,4,5,6-tetrahydro-7*H*-indole-7-one (8b). This compound was obtained from **7b** as a brown oil (0.93 g, 80%): $R_f = 0.62$ (CH_2Cl_2/EtOAc, 95:5); ^1H NMR ($CDCl_3$): $\delta =$ 2.50 (t, $J = 6.5$ Hz, 2H, CH_2), 2.71 (t, $J = 6.5$ Hz, 2H, CH_2), 5.57 (s, 2H, CH_2), 6.03 (d, $J = 2.2$ Hz, 1H, H-3), 6.85 (d, $J = 2.2$ Hz, 1H, H-2), 7.17–7.32 (m, 6H, Ar and CH), 13.62 ppm (d, $J = 10.5$ Hz, 1H, OH); ^{13}C NMR ($CDCl_3$): $\delta = (CDCl_3)$: 23.3 (t), 26.0 (t), 52.0 (t), 107.8 (d), 109.8 (s), 127.3 (2×d), 127.6 (d), 127.7 (s), 128.7 (2×d), 130.5 (d), 136.9 (s), 138.0 (s), 159.9 (d), 182.9 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3389$ (OH), 1630 cm^{-1} (CO); Anal. calcd (%) for $C_{16}H_{15}NO_2$ (253.30): C 75.87, H 5.97, N 5.53, found: C 76.02, H 5.85, N 5.92.

Ethyl 6-(hydroxymethylene)-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (8e). This compound was obtained from **7e** as a green solid (0.78 g, 68%): $R_f = 0.60$ (CH_2Cl_2/EtOAc, 95:5); mp: 78–79 °C; ^1H NMR ($CDCl_3$): $\delta = 1.36$ (t, $J = 7.1$ Hz, 3H, CH_3), 2.49 (t, $J = 6.5$ Hz, 2H, CH_2), 2.68 (t, $J = 6.5$ Hz, 2H, CH_2), 4.28–4.46 (m, 5H, CH_2 and CH_3), 6.72 (s, 1H, H-3), 7.28 (d, $J = 8.1$ Hz, 1H, CH), 13.75 ppm (d, $J = 8.1$ Hz, 1H, OH); ^{13}C NMR ($CDCl_3$): $\delta = 14.3$ (q), 22.8 (t), 25.6 (t), 34.7 (q), 60.7 (t), 110.4 (s), 114.2 (d), 129.0 (s), 130.9 (s), 133.4 (s), 161.0 (s), 162.4 (d), 184.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu} =$ 3390 (OH), 1707 (CO), 1631 (CO) cm^{-1}; Anal. calcd (%) for $C_{13}H_{15}NO_4$ (249.27): C 62.64, H 6.07, N 5.62, found: C 62.85, H 5.88, N 5.40.

Ethyl 1-benzyl-6-(hydroxymethylene)-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (8f). This compound was obtained from **7f** as a brown solid (0.97 g, 65%): $R_f = 0.64$ (CH_2Cl_2/EtOAc, 9:1); mp: 112–113 °C; ^1H NMR ($CDCl_3$): $\delta = 1.29$ (t, $J = 7.0$ Hz, 3H, CH_3), 2.48 (t, $J = 6.5$ Hz, 2H, CH_2), 2.71 (t, $J = 6.5$ Hz, 2H, CH_2), 6.14 (s, 2H, CH_2), 6.80–7.28 (m, 6H, Ar and CH), 13.68 ppm (d, $J = 7.8$ Hz, 1H, OH); ^{13}C NMR ($CDCl_3$): $\delta = 14.2$ (q), 22.8 (d), 25.5 (d), 49.5 (d), 60.7 (d), 110.3 (s), 115.2 (d), 126.6 (2×d), 127.0 (d), 128.4 (2×d), 128.6 (s), 130.6 (s), 134.0 (s), 138.7 (s), 160.6 (s), 162.4 (d), 183.7 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3389$ (OH), 1711 (CO), 1630 cm^{-1} (CO); Anal. calcd (%) for $C_{19}H_{19}NO_4$ (325.36): C 70.14, H 5.89, N 4.30, found: C 69.87, H 6.08, N 4.52.

General procedure for the synthesis of pyrrolo[3,2-*h*]quinazolines-2-ones 9a,b,e,f. To a suspension of the suitable hydroxymethylene derivative **8a,b,e,f** (3 mmol) in anhydrous EtOH, urea (0.36 g, 6 mmol) was added under N_2, and the mixture was heated at reflux for 24 h. The solid formed was filtered and purified by recrystallization or flash chromatography with CH_2Cl_2/EtOAc (7:3) as eluent.

9-Methyl-3,5,6,9-tetrahydro-2*H*-pyrrolo[3,2-*h*]quinazoline-2-one (9a). This product was obtained from **8a** as a yellow solid (0.37 g, 62%): $R_f = 0.41$ (CH_2Cl_2/EtOAc, 1:1); mp: 221–222 °C; ^1H NMR ([D_6]DMSO): $\delta =$ 2.57–2.67 (m, 4H, 2×CH_2), 3.89 (s, 3H, CH_3), 5.97 (d, $J = 2.4$ Hz, 1H, H-7), 7.05 (d, $J = 2.4$ Hz, 1H, H-8), 7.22 (d, $J =$ 11.1 Hz, 1H, H-4), 10.50 ppm (d, $J = 11.1$ Hz, 1H, NH); ^{13}C NMR ([D_6]DMSO): $\delta = 23.3$ (t), 29.8 (t), 36.0 (q), 106.2 (d), 108.6 (s), 127.7 (s), 131.1 (d), 134.3 (d), 134.9 (s), 154.5 (s), 180.3 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3402$ (NH), 1645 cm^{-1} (CO); Anal. calcd (%) for $C_{11}H_{11}N_3O$ (201.23): C 65.66, H 5.51, N 20.88, found: C 65.72, H 5.38, N 20.60.

9-Benzyl-3,5,6,9-tetrahydro-2*H*-pyrrolo[3,2-*h*]quinazoline-2-one (9b). This product was obtained from **8b** after 24 h at reflux as yellow solid (0.54 g, 65%): $R_f = 0.26$ (CH_2Cl_2/EtOAc, 1:1); mp: 144–145 °C; ^1H NMR ([D_6]DMSO): $\delta =$ 2.57–2.70 (m, 4H, 2×CH_2), 5.60 (s, 2H, CH_2), 6.07 (d, $J = 2.5$ Hz, 1H, H-7), 7.11–7.36 (m, 7H, ArH, H-8 and H-4), 10.41 ppm (d, $J = 11.1$ Hz, 1H, NH); ^{13}C NMR ([D_6]DMSO): $\delta = 23.8$ (t), 30.4 (t), 51.2 (t), 107.6 (d), 109.1 (s), 127.1 (2×d), 127.58 (s), 127.61 (d), 128.9 (2×d), 131.1 (d), 135.0 (d), 136.0 (s), 139.6 (s), 155.0 (s), 180.7 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3406$ (NH), 1648 cm^{-1} (CO); Anal. calcd (%) for $C_{17}H_{15}N_3O$ (277.33): C 77.63, H 5.45, N 15.15, found: C 77.80, H 5.15, N 15.30.

Ethyl 9-methyl-3,5,6,9-tetrahydro-2*H*-pyrrolo[3,2-*h*]quinazoline-2-one-8-carboxylate (9e). This product was obtained from **8e** as a yellow solid (0.49 g, 68%): $R_f = 0.26$ (CH_2Cl_2/EtOAc, 7:3); mp: 259–261 °C; ^1H NMR ([D_6]DMSO): $\delta = 1.29$ (t, $J = 7.1$ Hz, 3H, CH_3), 2.55–2.61 (m, 4H, 2×CH_2), 4.20 (s, 3H, CH_3), 4.27 (q, $J = 7.1$ Hz, 2H, CH_2), 6.72 (s, 1H, H-7), 7.35 (d, $J = 11.3$ Hz, 1H, H-4), 10.58 ppm (d, $J =$ 11.3 Hz, 1H, NH); ^{13}C NMR ([D_6]DMSO): $\delta = 14.1$ (q), 22.7 (t), 29.1 (t), 34.2 (q), 60.3 (t), 108.6 (s), 113.9 (d), 127.3 (s), 131.9 (s), 132.3 (s), 136.5 (d), 154.3 (s), 160.3 (s), 181.4 ppm (s); IR ($CHBr_3$): $\tilde{\nu} = 3409$ (NH), 1711 (CO), 1647 cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{15}N_3O_3$ (273.29): C 61.53, H 5.53, N 15.38, found: C 61.32, H 5.74, N, 15.02.

Ethyl 9-benzyl-3,5,6,9-tetrahydro-2*H*-pyrrolo[3,2-*h*]quinazoline-2-one-8-carboxylate (9f). This product was obtained from **8f** as a yellow solid (0.63 g, 60%): $R_f = 0.42$ (CH_2Cl_2/EtOAc, 7:3); mp: 223–224 °C; ^1H NMR ([D_6]DMSO): $\delta = 1.19$ (t, $J = 7.1$ Hz, 3H, CH_3), 2.61–2.70 (m, 4H, 2×CH_2), 4.17 (q, $J = 7.1$ Hz, 2H, CH_2), 6.11 (s, 2H, CH_2), 6.75 (s, 1H, H-7), 6.92–7.39 (m, 6H, Ar and H-4), 10.47 ppm (d, $J =$ 11.4 Hz, 1H, NH); ^{13}C NMR ([D_6]DMSO): $\delta = 14.0$ (q), 22.7 (t), 29.1 (t),

48.7 (t), 60.3 (t), 108.5 (s), 115.0 (d), 125.6 (2×d), 126.7 (d), 126.9 (s), 128.4 (2×d), 131.7 (s), 133.0 (s), 136.8 (d), 139.1 (s), 154.3 (s), 160.0 (s), 181.3 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3404 (NH), 1718 (CO), 1644 cm^{-1} (CO); Anal. calcd for $C_{20}H_{19}N_3O_3$ (349.39): C 68.75, H 5.48, N 12.03, found: C 68.58, H 5.60, N 12.34.

General procedure for the synthesis of 6-[(dimethylamino)-methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-ones (10a–c,e–g). To a solution of the suitable ketone **7a–c,e–g** (3 mmol) in anhydrous DMF (2 mL), TBDMAM (0.94 mL, d = 0.844 g mL^{-1}, 4.5 mmol) was added, and the mixture was stirred under microwave irradiation (CEM Discover Labmate™ apparatus; power = 150 W, t = 5–15 min, p = 690 kPa, T = 190 °C). The reaction mixture was poured onto crushed ice, and the solid formed dried to give the pure compounds **10** with the sole exception of 4-methoxybenzyl-substituted derivatives **10c** and **10g** which were unstable, could not be isolated as pure compounds, and were used as crude products for the successive step. The elemental analyses, NMR, and IR spectra were in accordance with compounds obtained by conventional methods previously reported by us.[7]

6-[(Dimethylamino)methylene]-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (10a). This compound was obtained from **7a** (5 min) as a brown solid (0.59 g, 96%): R_f = 0.50 (CH_2Cl_2/EtOAc, 9:1); mp: 49–51 °C.

1-Benzyl-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one (10b). This compound was obtained from **7b** (15 min) as a brown solid (0.82 g, 98%): R_f = 0.48 (CH_2Cl_2/EtOAc, 9:1); mp: 116–118 °C.

6-[(Dimethylamino)methylene]-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (10c). This compound was obtained from **7c** (5 min) as a dark-brown oil and used directly in the next step.

Ethyl 6-[(dimethylamino)methylene]-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (10e). This compound was obtained from **7e** (5 min) as a brown solid (0.81 g, 98%): R_f = 0.52 (CH_2Cl_2/EtOAc, 9:1); mp: 50–52 °C.

Ethyl 1-benzyl-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (10f). This compound was obtained from **7f** (5 min) as a brown solid (1.1 g, 100%); R_f = 0.56 (CH_2Cl_2/EtOAc, 9:1); mp: 101–103 °C.

Ethyl 6-[(dimethylamino)methylene]-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (10g). This compound was obtained from 7 g (5 min) as a dark-brown oil and used directly in the next step.

General procedure for the synthesis of 2-aminopyrrolo[3,2-*h*]quinazolines (11a–c,e–g). To a suspension of NaOMe (0.81 g, 15.0 mmol) in anhydrous EtOH (20 mL), guanidine nitrate (0.92 g, 7.5 mmol) was added. A solution of the suitable dialkylamino derivative **10** (1.5 mmol) in anhydrous EtOH (15 mL) was added dropwise to the suspension, and the mixture was heated at reflux. The mixture was poured onto crushed ice, extracted with EtOAc (3× 150 mL), and the organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by flash chromatography eluting with CH_2Cl_2/EtOAc (9:1).

2-Amine-9-methyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (11a). This product was obtained from **10a** (2 h) a brown solid (0.20 g, 67%): R_f = 0.19 (CH_2Cl_2/EtOAc, 1:1); mp: 134–135 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.50–2.61 (m, 4H, 2×CH_2), 4.02 (s, 3H, CH_3), 5.95 (d, J = 2.5 Hz, 1H, H-7), 6.14 (s, 2H, NH_2), 6.87 (d, J = 2.5 Hz, 1H, H-8), 7.91 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 21.8 (t), 24.9 (t), 36.2 (q), 106.3 (d), 114.7 (s), 124.8 (s), 128.0 (d), 128.2 (s), 154.4 (d), 155.6 (s), 162.1 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3558–3406 (NH_2) cm^{-1}; Anal. calcd for $C_{11}H_{12}N_4$ (200.24): C 65.98, H 6.04, N 27.98, found: C 66.15, H 5.80, N 28.30.

2-Amine-9-benzyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (11b). This product was obtained from **10b** (2 h) as a brown solid (0.20 g, 48%): R_f = 0.16 (CH_2Cl_2/EtOAc, 7:3); mp: 121–122 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 3.18–3.23 (m, 4H, 2×CH_2), 5.82 (s, 2H, CH_2), 6.01 (d, J = 2.6 Hz, 1H, H-7), 6.20 (s, 2H, NH_2), 7.06 (d, J = 2.6 Hz, 1H, H-8), 7.19–7.31 (m, 5H, Ar), 7.89 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 21.8 (t), 24.7 (t), 50.8 (t), 107.0 (d), 114.5 (s), 124.1 (s), 127.0 (d), 127.3 (2×d), 127.6 (d), 128.3 (2×d), 128.8 (s), 139.5 (s), 154.5 (d), 155.3 (s), 162.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3500–3408 cm^{-1} (NH_2); Anal. calcd (%) for $C_{17}H_{16}N_4$ (276.34): C 73.89, H 5.84, N 20.27, found: C 74.04, H 5.63, N 20.50.

2-Amine-9-(4-methoxybenzyl)-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (11c). This product was obtained from **10c** (3 h) as a brown solid (0.32 g, 70%); R_f = 0.19 (CH_2Cl_2/EtOAc, 7:3); mp: 94–95 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.46–2.59 (m, 4H, 2×CH_2), 3.68 (s, 3H, OMe), 5.72 (s, 2H, CH_2), 5.99 (d, J = 2.6 Hz, 1H, H-7), 6.24 (s, 2H, NH_2), 6.83 (d, J = 8.6 Hz, 2H, H-3′ and H-5′), 7.05 (d, J = 2.6 Hz, 1H, H-8), 7.20–7.25 (d, J = 8.6 Hz, 2H, H-2′ and H-6′), 7.89 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 21.8 (t), 24.8 (t), 50.2 (t), 54.9 (q), 106.9 (d), 113.6 (2×d), 114.5 (s), 124.0 (s), 127.4 (d), 128.8 (s), 128.9 (2×d), 131.4 (s), 154.5 (d), 155.4 (s), 158.4 (s), 162.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3515–3410 cm^{-1} (NH_2); Anal. calcd (%) for $C_{18}H_{18}N_4O$ (306.37): C 70.57, H 5.92, N 18.29, found: C 70.45, H 6.18, N 18.38.

Ethyl 2-amino-9-methyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (11e). This product was obtained from **10e** (2 h) as a white solid (0.28 g, 68%); R_f = 0.23 (CH_2Cl_2/EtOAc, 7:3); mp: 235–237 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.28 (t, J = 7.1 Hz, 3H, CH_3), 2.51–2.63 (m, 4H, 2×CH_2), 4.24 (q, J = 7.1 Hz, 2H, CH_2), 4.39 (s, 3H, CH_3), 6.42 (s, 2H, NH_2), 6.76 (s, 1H, H-7), 8.07 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 14.2 (q), 21.2 (t), 24.5 (t), 34.7 (q), 59.7 (t), 99.5 (s), 114.7 (d), 116.7 (s), 125.2 (s), 126.5 (s), 154.8 (s), 156.1 (d), 160.4 (s), 162.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3305–3185 (NH_2), 1695 cm^{-1} (CO); Anal. calcd (%) for $C_{14}H_{16}N_4O_2$ (272.31): C 61.75, H 5.92, N 20.57, found: C 62.02, H 5.76, N 20.70.

Ethyl 2-amino-9-benzyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (11f). This product was obtained from **10f** (2 h) as a white solid (0.32 g, 62%); R_f = 0.28 (CH_2Cl_2/EtOAc, 7:3); mp: 175–177 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.22 (t, J = 7.1 Hz, 3H, CH_3), 2.49–2.66 (m, 4H, 2×CH_2), 4.19 (q, J = 7.1 Hz, 2H, CH_2), 6.38 (s, 2H, NH_2), 6.46 (s, 2H, CH_2), 6.86 (s, 1H, H-7), 7.02 (d, J = 7.3 Hz, 2H, H-2′ and H-6′), 7.15–7.28 (m, 3H, H-3′ H-4′ and H-5′), 8.05 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 14.1 (q), 21.1 (t), 24.3 (t), 48.5 (t), 59.8 (t), 115.9 (d), 116.5 (s), 125.0 (s), 126.3 (2×d), 126.7 (d), 127.2 (s), 128.2 (2×d), 130.5 (s), 139.5 (s), 154.6 (s), 156.2 (d), 160.2 (s), 162.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3519–3409 (NH_2), 1699 (CO) cm^{-1}; Anal. calcd (%) for $C_{20}H_{20}N_4O_2$ (348.40): C 68.95, H 5.79, N 16.08, found: C 69.04, H 5.65, N 16.38.

Ethyl 2-amino-9-(4-methoxybenzyl)-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (11g). This product was obtained from **10g** (2 h) as a white solid (0.44 g, 78%); R_f = 0.32 (CH_2Cl_2/EtOAc, 7:3); mp: 173–175 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.25 (t, J = 7.1 Hz, 3H, CH_3), 2.49–2.52 (m, 4H, 2×CH_2), 3.66 (s, 3H, CH_3), 4.21 (q, J = 7.1 Hz, 2H, CH_2), 6.38–6.44 (m, 4H, CH_2 and NH_2), 6.76 (s, 1H, H-7), 6.82 (d, J = 8.6 Hz, 2H, H-3′ and H-5′), 6.99–7.02 (d, J = 8.6 Hz, 2H, H-2′ and H-6′), 8.06 ppm (s, 1H, H-4); ^{13}C NMR ([D$_6$]DMSO): δ = 14.1 (q), 21.1 (t), 24.4 (t), 47.7 (t), 54.9 (q), 59.9 (t), 113.6 (2×d), 115.8 (d), 116.5 (s), 124.9 (s), 127.2 (s), 127.9 (2×d), 130.4 (s), 131.4

(s), 154.7 (s), 156.2 (d), 158.1 (s), 160.3 (s), 162.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3456–3409 (NH_2), 1695 cm^{-1} (CO); Anal. calcd for $C_{21}H_{22}N_4O_3$ (378.43): C 66.65, H 5.86, N 14.81, found: C 66.47, H 6.10, N 15.03.

General procedure for the synthesis of 2-anilino-pyrrolo[3,2-*h*]quinazolines 12a-c,e-g. To a suspension of the suitable dimethylamino derivative **10** (1.5 mmol) in anhydrous DMF (7 mL), phenylguanidine (0.61 g, 4.5 mmol) was added. The mixture was heated at reflux for 15–90 min, then cooled and poured onto crushed ice. The formed precipitate was filtered and recrystallized from EtOH.

2-Anilino-9-methyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (12a). This product was obtained from **10a** (15 min) as a white solid (0.19 g, 46%): R_f = 0.45 (CH_2Cl_2/EtOAc, 95:5); mp: 196–197 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.60–2.77 (m, 4H, 2×CH_2), 4.06 (s, 3H, CH_3), 6.00 (d, J = 2.5 Hz, 1H, H-7), 6.87–6.95 (m, 2H, H-8 and H-4′), 7.26 (t, J = 7.5 Hz, 2H, H-3′ and H-5′), 7.73 (d, J = 7.4 Hz, 2H, H-2′ and H-6′), 8.14 (s, 1H, H-4), 9.20 ppm (s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 21.7 (t), 24.9 (t), 36.5 (q), 106.5 (d), 117.0 (s), 118.7 (2×d), 120.7 (d), 124.8 (s), 128.3 (2×d), 128.7 (d), 129.1 (s), 140.9 (s), 154.1 (d), 155.2 (s), 158.6 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3419 cm^{-1} (NH); Anal. calcd (%) for $C_{17}H_{16}N_4$ (276.34): C 73.89, H 5.84, N 20.27, found: C 74.07, H 5.60, N 20.03.

2-Anilino-9-benzyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (12b). This product was obtained from **10b** (15 min) as a white solid (0.67 g, 67%): R_f = 0.48 (CH_2Cl_2/EtOAc, 95:5); mp: 189–191 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.53–2.70 (m, 4H, 2×CH_2), 5.88 (s, 2H, CH_2), 6.08 (d, J = 2.6 Hz, 1H, H-7), 6.87 (t, J = 7.6 Hz, 1H, H-4′), 7.06–7.31 (m, 8H, Ar and H-8), 7.60 (d, J = 7.6 Hz, 2H, H-2′ and H-6′), 8.14 (s, 1H, H-4), 9.20 ppm (s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 21.6 (t), 24.9 (t), 50.7 (t), 107.5 (d), 117.0 (s), 118.9 (2×d), 120.9 (d), 124.3 (s), 126.8 (2×d), 127.1 (d), 128.2 (d), 128.3 (2×d), 128.4 (2×d), 129.6 (s), 139.5 (s), 140.8 (s), 154.3 (d), 155.1 (s), 158.5 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3419 cm^{-1} (NH); Anal. calcd for $C_{23}H_{20}N_4$ (352.44): C 78.38, H 5.72, N 15.90, found: C 78.02, H 5.56, N 16.12.

2-Anilino-9-(4-methoxybenzyl)-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (12c). This product was obtained from **10c** (1 h) as a white solid (0.26 g, 70%): R_f = 0.45 (CH_2Cl_2/EtOAc, 95:5); mp: 178–180 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.52–2.69 (m, 4H, 2×CH_2), 3.67 (s, 3H, CH_3), 5.78 (s, 2H, CH_2), 6.04 (d, J = 2.6 Hz, 1H, H-7), 6.79–6.92 (m, 3H, H-4′ and Ar), 7.04–7.22 (m, 5H, H-8 and Ar), 7.65 (d, J = 7.7 Hz, 2H, H-2′ and H-6′), 8.13 (s, 1H, H-4), 9.23 ppm (s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 21.6 (t), 24.9 (t), 50.2 (t), 54.9 (q), 107.3 (d), 113.7 (2×d), 117.0 (s), 118.9 (2×d), 120.9 (d), 124.1 (s), 128.06 (d), 128.29 (2×d), 128.32 (2×d), 129.6 (s), 131.3 (s), 140.8 (s), 154.2 (d), 155.2 (s), 158.3 (s), 158.6 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3417 cm^{-1} (NH); Anal. calcd (%) for $C_{24}H_{22}N_4O$ (382.46): C 75.37, H 5.80, N 14.65, found: C 75.63, H 5.64, N 14.88.

Ethyl 2-anilino-9-methyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (12e). This product was obtained from **10e** (1.5 h) as a white solid (0.26 g, 70%): R_f = 0.59 (CH_2Cl_2/EtOAc, 95:5); mp: 204–206 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.29 (t, J = 7.1 Hz, 3H, CH_3), 2.64–2.70 (m, 4H, 2×CH_2), 4.25 (q, J = 7.1 Hz, 2H, CH_2), 4.40 (s, 3H, CH_3), 6.80 (s, 1H, H-7), 6.93 (t, J = 7.8 Hz, 1H, H-4′), 7.29 (t, J = 7.8 Hz, 2H, H-3′ and H-5′), 7.71 (d, J = 7.8 Hz, 2H, H-2′ and H-6′), 8.30 (s, 1H, H-4), 9.41 ppm (1H, s, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 14.2 (q), 21.0 (t), 24.5 (t), 34.9 (q), 59.9 (t), 114.8 (d), 118.9 (2×d), 119.0 (s), 121.1 (d), 125.7 (s), 127.1 (s), 128.4 (2×d), 130.7 (s), 140.6 (s), 154.5 (s), 155.8 (d), 158.5 (s), 160.4 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3417 (NH), 1697 cm^{-1} (CO); Anal. calcd for $C_{20}H_{20}N_4O_2$ (348.40): C 68.95, H 5.79, N 16.08, found: C 69.22, H 5.58, N 15.83.

Ethyl 2-anilino-9-benzyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (12f). This product was obtained from **10f** (2 h) as a yellow solid (0.27 g, 65%): R_f = 0.54 (CH_2Cl_2/EtOAc, 95:5); mp: 206–207 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.20 (t, J = 7.1 Hz, 3H, CH_3), 2.69–2.75 (m, 4H, 2×CH_2), 4.16 (q, J = 7.1 Hz, 2H, CH_2), 6.45 (s, 2H, CH_2Ar), 6.84–6.93 (m, 4H, Ar), 7.08–7.29 (m, 5H, H-7 and Ar), 7.48–7.52 (m, 2H, Ar), 8.30 (s, 1H, H-4), 9.38 ppm (s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 14.2 (q), 21.0 (t), 24.6 (t), 48.7 (t), 60.0 (t), 108.3 (s), 116.0 (d), 119.0 (d), 121.3 (d), 125.4 (s), 126.0 (d), 126.8 (d), 127.8 (s), 128.4 (2×d), 130.5 (s), 139.5 (s), 140.4 (s), 154.5 (s), 156.2 (d), 158.3 (s), 160.0 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3418 (NH), 1700 cm^{-1} (CO); Anal. calcd (%) for $C_{26}H_{24}N_4O_2$ (424.19): C 73.56, H 5.70, N 13.20, found: C 73.74, H 5.44, N 13.52.

Ethyl 2-anilino-9-(4-methoxybenzyl)-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline-8-carboxylate (12g). This product was obtained from **10g** (30 min) as a yellow solid (0.48 g, 70%): R_f = 0.56 (CH_2Cl_2/EtOAc, 9:1); mp: 157–158 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.20 (t, J = 7.1 Hz, 3H, CH_3), 2.60–2.73 (m, 4H, 2×CH_2), 3.65 (s, 3H, CH_3), 4.18 (q, J = 7.1 Hz, 2H, CH_2), 6.38 (s, 2H, CH_2), 6.76–6.93 (m, 6H, H-7 and Ar), 7.16 (t, J = 7.8 Hz, 2H, H-3′ and H-5′), 7.55 (d, J = 7.8 Hz, 2H, H-2′ and H-6′), 8.30 (s, 1H, H-4), 9.41 ppm (s, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): δ = 14.1 (q), 21.0 (t), 24.5 (t), 47.9 (t), 54.9 (q), 59.9 (t), 99.5 (s), 113.7 (2×d), 116.0 (d), 119.0 (2×d), 121.2 (d), 125.3 (s), 127.4 (2×d), 127.8 (s), 128.3 (2×d), 130.3 (s), 131.3 (s), 140.4 (s), 154.5 (s), 156.0 (d), 158.1 (s), 158.5 (s), 160.1 ppm (s); IR ($CHBr_3$): $\tilde{\nu}$ = 3417 (NH), 1699 cm^{-1} (CO); Anal. calcd for $C_{27}H_{26}N_4O_3$ (454.53): C 71.35, H 5.77, N 12.33, found: C 70.98, H 5.94, N 12.00.

General procedure for the synthesis of pyrrolo[3,2-*h*]quinazolines 13b,d. To a suspension of the suitable ketone **7b,d** (2.14 mmol) and *p*-toluenesulfonic acid (0.1 g, 0.5 mmol) in formamide (10 mL) under N_2, tris(formylamino)methane (0.62 g, 4.28 mmol) was added and the mixture was heated at reflux. After 24 h it was poured onto crushed ice, the formed precipitate, filtered under vacuum, was purified by flash chromatography eluting with CH_2Cl_2/EtOAc (9:1).

9-Benzyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (13b). This product was obtained from **7b** as a yellow solid (0.25 g, 45%): R_f = 0.30 (CH_2Cl_2/EtOAc, 9:1); mp: 67–68 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.67–2.88 (m, 4H, 2×CH_2), 5.82 (s, 2H, CH_2), 6.10 (d, J = 2.6 Hz, 1H, H-7), 7.14–7.33 (m, 6H, Ar and H-8), 8.39 (s, 1H, H-4), 8.78 ppm (s, 1H, H-2); ^{13}C NMR ([D$_6$]DMSO): δ = 21.0 (t), 25.3 (t), 50.9 (t), 107.6 (d), 123.7 (s), 126.3 (s), 126.8 (2×d), 127.1 (d), 128.4 (2×d), 128.9 (d), 129.7 (s), 139.2 (s), 153.4 (d), 154.5 (s), 156.2 ppm (d); Anal. calcd (%) for $C_{17}H_{15}N_3$ (261.33): C 78.13, H 5.79, N 16.08, found: C 78.34, H 5.62, N 16.30.

9-Phenyl-6,9-dihydro-5*H*-pyrrolo[3,2-*h*]quinazoline (13d). This product was obtained from **7d** as a brown solid (0.28 g, 54%): R_f = 0.35 (CH_2Cl_2/EtOAc, 9:1); mp: 84–86 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.79–2.98 (m, 4H, 2×CH_2), 6.23 (d, J = 2.7 Hz, 1H, H-7), 6.97 (d, J = 2.7 Hz, 1H, H-8), 7.26–7.47 (m, 5H, Ar), 8.34 (s, 1H, H-4), 8.61 ppm (s, 1H, H-2); ^{13}C NMR ([D$_6$]DMSO): δ = 22.0 (t), 26.1 (t), 100.0 (s), 108.6 (d), 125.4 (2×d), 126.6 (s), 127.1 (d), 128.7 (2×d), 129.3 (d), 132.1 (s), 140.5 (s), 153.3 (d), 154.5 (s), 156.7 ppm (d); Anal. calcd (%) for $C_{16}H_{13}N_3$ (247.30): C 77.71, H 5.30, N 16.99, found: C 77.47, H 5.49, N 17.22.

Biology

Reagents. If not otherwise indicated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).

Cell lines. Jurkat (human T-cell leukemia) and K-562 (human chronic myeloid leukemia) cells were cultured in RPMI-1640 medium; LoVo (human intestinal adenocarcinoma) cells were grown in Ham's F-12 medium, and MCF-7 (breast adenocarcinoma) and A-431 (EGFR-overexpressing vulvar squamous cell carcinoma) cells were cultured in DMEM. All media were supplemented with penicillin G (115 U mL^{-1}), streptomycin (115 μg mL^{-1}), and 10% fetal bovine serum (Invitrogen, Milan, Italy).

Irradiation procedure. HPW 125 Philips lamps (Wood's lamps), emitting mainly at $\lambda = 365$ nm, were used for irradiation experiments. The spectral irradiance of the source was 4.0 mW cm^{-2} as measured by a radiometer (Cole–Parmer Instrument Company, Niles, IL, USA) equipped with a 365-CX sensor.

Spectrophotometric measurements. UV/Vis absorption spectra were recorded on a PerkinElmer Lambda 15 spectrophotometer, and emission spectra were recorded on a PerkinElmer LS-50B fluorimeter.

Cellular antiproliferative tests and phototoxicity. Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 μL complete medium containing 5×10^3 cells. Plates were harvested at 37 °C in a humidified incubator containing 5% CO_2 for 24 h prior to cell viability experiments. Drugs were dissolved in DMSO and were then diluted with Hank's balanced salt solution (HBSS, pH 7.2) for phototoxicity experiments or in the appropriate complete medium for the cytotoxicity assays. In cytotoxicity tests after medium removal, 100 μL drug solution at various concentrations were added to each well and incubated at 37 °C for 72 h. In phototoxicity experiments after medium removal, 100 μL drug solution were put into each well and incubated at 37 °C for 30 min and then irradiated (2.5 and 3.75 J cm^{-2}). After irradiation, the drug solution was replaced by cell culture medium, and plates were incubated for 72 h. In both cases, cell viability was assayed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) test.[14]

Cell-cycle analysis. For flow cytometric analysis of DNA content, 5×10^5 Jurkat cells in exponential growth were treated with or without test compound for 24 h. Cells were centrifuged (8 min, 4 °C, 300 *g*) and fixed with ice-cold EtOH (70%), treated with lysis buffer containing RNase A, and then stained with propidium iodide (PI). Samples were analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson, USA). DNA histograms were examined using WinMDI 2.9 (Windows multiple document interface for flow cytometry).

Externalization of phosphatidylserine. Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured with a BD FACSCalibur flow cytometer by adding Annexin V–FITC to cells according to the manufacturer's instructions (Annexin V Fluos, Roche Diagnostics, Indianapolis, IN, USA). Cells were simultaneously stained with PI. Excitation was set at $\lambda = 488$ nm, and the emission filters were at $\lambda = 525$ and 585 nm.[15]

Assessment of mitochondrial changes. The mitochondrial membrane potential was measured with the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1, Molecular Probes, USA), as described elsewhere.[18] Briefly, 3 h post-irradiation, Jurkat cells were collected by centrifugation (5 min, 37 °C, 300 *g*) and resuspended in HBSS containing JC-1 at 1 μM. Cells were then incubated at 37 °C for 10 min, and fluorescence was examined in FL1 and FL2. The production of ROS was measured by flow cytometry using the fluorescence probes HE and DCFDA (both from Molecular Probes, USA); 6 h after irradiation, Jurkat cells were incubated in HBSS containing HE or DCFDA at concentrations of 2.5 and 5 μM, respectively. Cells were then incubated at 37 °C for 30 min, centrifuged (5 min, 37 °C, 300 *g*), and resuspended in HBSS. The fluorescence was recorded directly with the flow cytometer by using excitation at $\lambda = 488$ nm and emission at $\lambda = 585$ and 530 nm for HE and DCFDA, respectively.[19]

Lysosomal stability assessment. Jurkat cells were assessed for lysosomal stability using the acridine orange (AO) uptake method.[22] After 3 h from the irradiation in the presence of test compounds, cells were stained with AO at 5 μg mL^{-1} at 37 °C for 15 min, washed, and then analyzed by flow cytometry using excitation at $\lambda = 488$ nm and detecting the emission at $\lambda = 546$ nm.

Lipid peroxidation. Jurkat cells (5×10^5) were irradiated (2.5 J cm^{-2}) in the presence of the most active compounds and then incubated for 24 h. To verify lipid peroxidation, 900 μL supernatant were collected and put at 253 K after having added 90 μL 2,6-di-*tert*-butyl-*p*-cresol (BHT, 2% in absolute EtOH). Cells were washed, resuspended in 500 μL H_2O, and 400 μL cells were lysed with 400 μL sodium dodecyl sulfate (SDS, 1% in H_2O); 300 μL of this latter suspension were used for protein quantification by the Peterson method.[26] Lipid peroxidation was measured in the supernatant fraction using a thiobarbituric acid (TBA) assay as described by Morlière et al.[23] A standard curve of 1,1,3,3-tetraethoxypropane was used to quantify the amount of malondialdehyde produced. Data were expressed as nmol TBARS normalized to the total protein content in each sample.

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Keywords: angelicin heteroanalogues · antitumor agents · apoptosis · photochemotherapeutic agents · pyrrolo[3,2-*h*]quinazolines

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