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Research paper

Pyrazolo[3,4-*h*]quinolines promising photosensitizing agents in the treatment of cancer

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ABSTRACT

A new series of pyrazolo[3,4-*h*]quinolines, heteroanalogues of angelicin was conveniently prepared with a broad substitution pattern. A large number of derivatives was obtained and the cellular photocytotoxicity was evaluated *in vitro* against 5 different human tumor cell lines with GI_{50} values reaching the nanomolar level (14.52–0.04 μM). Selected compounds were able to photoinduce a massive cell death with the involvement of mitochondria. Their photodamage cellular targets were proteins and lipids and they did not cause any kind of DNA photodamage. This latter event is of considerable importance in the modulation of long term side effects, generally associated with the use of classical furocoumarins.

1. Introduction

Cancer drug development remains a high unmet medical need, thus the discovery of small molecules as cancer drugs is a challenging aspect of the drug discovery [1–6].

The therapeutic properties of light have been known for thousands of years, but it was only in the last century that photodynamic therapy (PDT) was developed. PDT requires topycal or systemic administration of a drug called photosensitizer, followed by light activation of proper wavelength in accordance with the absorption spectrum of the photosensitizer [7–9]. Generally, the controlled and targeted release of ROS by photosensitizer is crucial in PDT. Since its initial application, PDT continues to find increasing use in the treatment of certain types of malignancies including esophageal, head and neck, breast, prostate, bladder, skin and lung tumors [10,11].

The use of PDT in combination with conventional therapies [12,13] and selective photosensitizer delivery are more recent fields of investigations [14] and a great deal of research is focused on the development of more powerful photosensitizers that more specifically target cancer cells having mitochondria and endoplasmatic reticulum as preferential targets [15–18].

Furocoumarins are natural occurring photosensitizers which intercalate between the base pairs of DNA "in the dark" and photobind to it upon UV-A irradiation. Among them, 8-methoxypsoralen (8-MOP) was approved by FDA for the treatment of cutaneous T-cell lymphoma and in clinical trial for the treatment of Hodgkins and non-Hodgkins lymphomas. Considerable efforts have been done to develop monofunctional furocoumarins, which cannot crosslink with DNA, thereby reducing indesiderable side effects [19].

In this contest, the main research line dealt with the synthesis of

Abbreviations: AO, acridine orange; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HE, hydroethidine; JC1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine; MTT test, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test; PI, propidium iodide; PS, phosphatidylserine; RNAase, ribonuclease; ROS, reactive oxygen species; TBARS, thiobarbituric reactive substances.

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angelicin, an angular furocumarin, which, for geometrical reasons, prevents the formation of interstrand crosslinks (ISC) and its heteroanalogues (Chart 1), such as thioangelicins [20] and furoquinolinones [21].

Quinolinones are considered attractive scaffold in medicinal chemistry because of their variety of biological activities, including antibacterial [22], anticancer [23,24], antiviral [25] and antituberculosis properties [26]. However, not much is reported in literature about their capability of interacting with light and their possible use as photosensitizing agents.

In our continuing studies aimed at the discovery [27–40] and development of new potential anticancer drug candidates, we have devoted more than ten years in the search of new photoactivating agents [41,42] with improved photoantiproliferative activity dissociated from side effects. In this contest, we discovered new classes of small nitrogen heterocycles (**1**–**5**, Chart 1) [43–49]. Among sulfur derivatives, the best results were achieved with the class of thiopyrano[2,3-*e*]indol-2-ones **1**, which despite their remarkable phototoxicity a significative photodamage to lipids and proteins and photooxidation of DNA bases were observed.

Pyrroloquinolinones **3**–**5** demonstrated very promising features as photosensitizing agents. In fact, in addition to an higher UV-A dose dependent photoantiproliferative effect than the reference drugs 8-MOP, at variance of this latter, they did not affect DNA and mitochondria were their preferential target in the photoinduced cell death, with high production of ROS and an extensive oxidative damage to membrane lipids in accordance with the required properties of photoactivable drugs. A very attractive result was that pyrrolo[3,2-*h*]quinolin-2-ones **4** exert their phototoxicity without any DNA damage (no DNA strand breaks and no DNA oxidative damages), which is the main origin of the long term side effects of the PUVA therapy. These findings could represent a significant advantage for a possible use in therapy, as the main side effects of psoralens are genotoxicity and mutagenicity. In this paper, in an attempt to further explore the effect of the condensation of the quinoline moiety to the pyrazole ring, extensively represented in medicinal chemistry in the treatment of cancer [50–53], we reported the synthesis, the study of the mechanism of action and the biological activity of a new series of derivatives of the pyrazolo[3,4-*h*]quinoline ring system. Some of these compounds showed much higher activity than that of angelicin.

2. Chemistry

The synthetic pathway to obtain the tricyclic system pyrazolo[3,4-*h*]quinoline **10**–**13** is outlined in Scheme 1. On the basis of our previous experience on pyrroloquinolinones, 1,5,6,7-tetrahydro-4*H*-indazol-4-ones **8a-h** would be ideal precursors for our purposes, considering that they can be easily functionalized in α position to the carbonyl to give the access to enaminoketons **9a-h**. Such type of intermediates are recognized as versatile key intermediates. In fact, the annular carbonyl group and the exocyclic carbon in α position represent the two electrophilic centers, readily available for further cyclizations with various dinucleophiles [4,40,41,45–49,53] (Table 1).

Ketones **8a-h**, were prepared by convenient modifications of the known procedures [54–56], which led to an improvement of the yields (60–93%) of the desired products. Such reactions started from 2-[(dimethyl-amino)methylene]cyclohexane-1,3-dione **7**, in turn prepared from 1,3-cyclohexanedione **6** [39], and the properly substituted hydrazines in a 1:1 ratio, heating under reflux using a mixture of ethanol and acetic acid (2:1) as solvent, or alternatively only acetic acid when 2-, 3- and 4-chlorophenylhydrazines are used as nucleophiles.

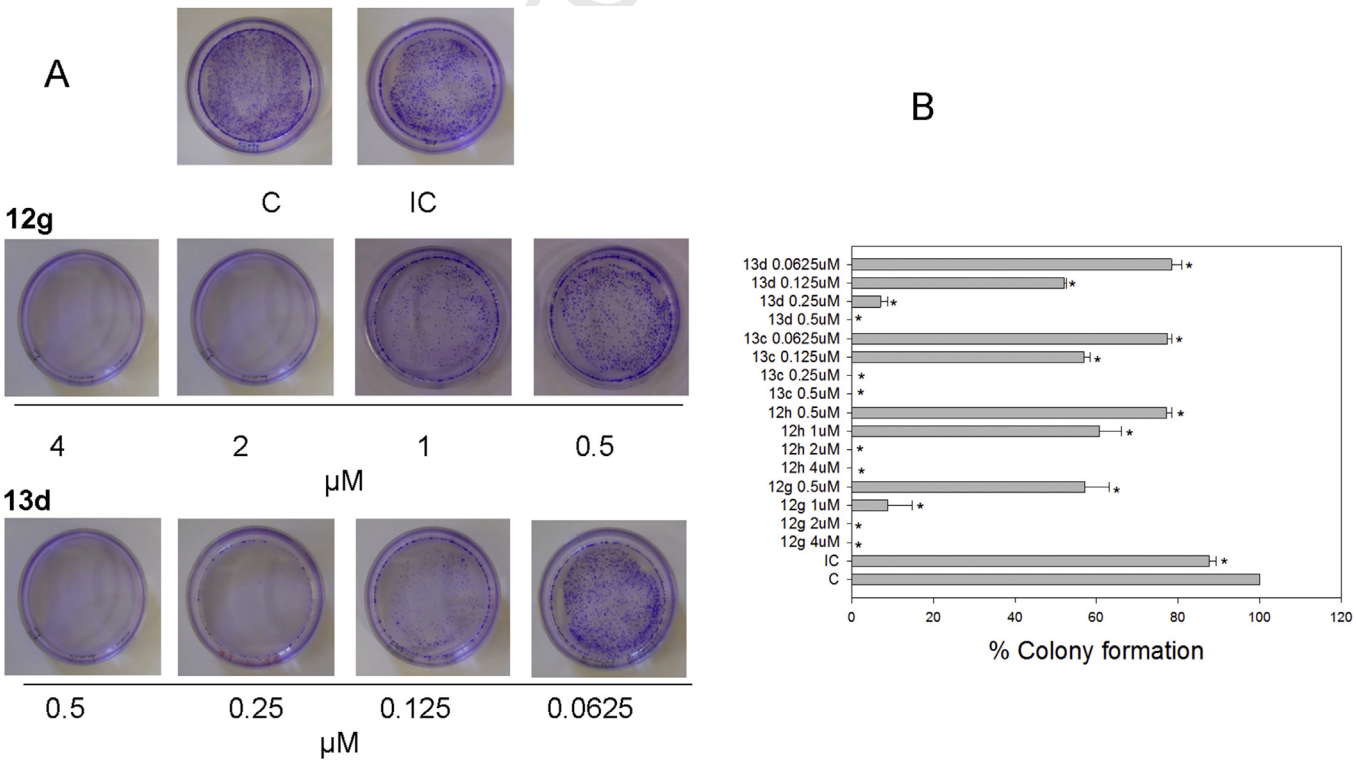

Fig. 1. Colony formation assays were performed as described in the Experimental Section. A) The images showed representative wells of A-549 colonies stained with crystal violet after irradiation with the indicated concentrations of **12g** or **13d**. B) Results were represented as percent colony formation relative to untreated cells (C = 100%); error bars represent the mean ± SEM of three independent experiments. C = not irradiated control; IC = irradiated control.

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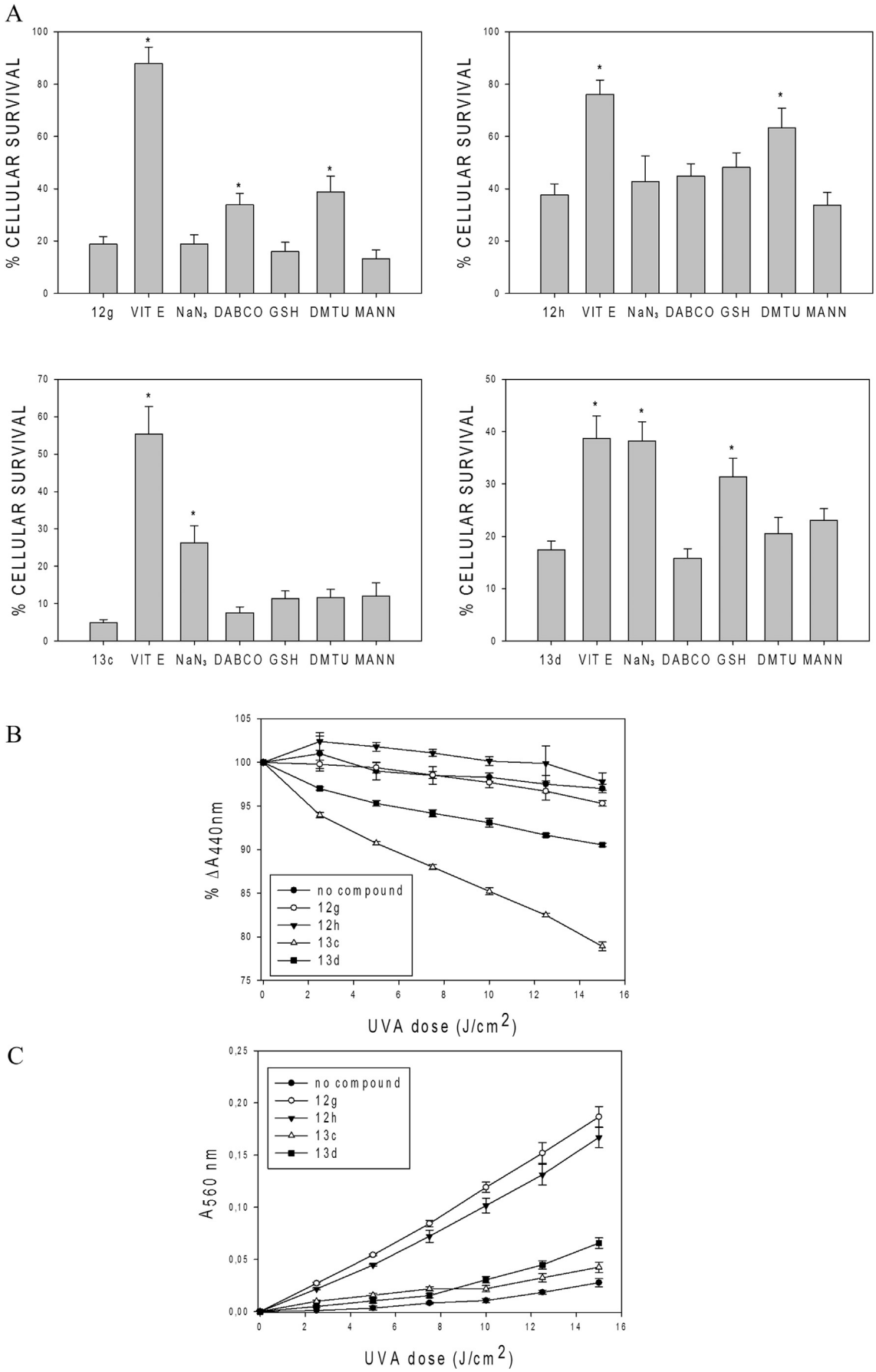

Fig. 2. A) Effect of different scavengers on the phototoxicity induced by compounds in Jurkat cells. Cell viability was assayed by MTT test after 72 h from the irradiation (2.5 J/cm^2) in the presence of the tested compounds (0.5 μM **12g** and **12h**; 0.2 μM **13c** and **13d**) and vitamin E (60 μM), NaN_3 (10 mM), DABCO (1 mM), GSH (1 mM), DMTU (1 mM) and Man (10 mM). B) RNO bleaching induced by singlet oxygen and C) increase in absorbance at 560 nm of NBT reduced by superoxide anion produced compounds under increasing UV-A doses. A sample without compounds was used as control.

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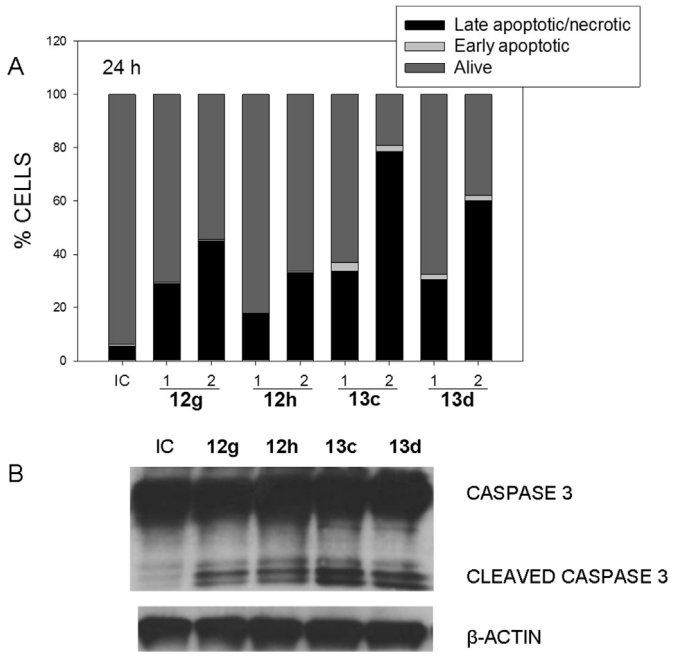

Fig. 3. A) Exposure of PS and analysis of PI staining 24 h after irradiation (2.5 J/cm^2) of Jurkat cells treated with compounds at 1 or 2 μM. Cells were stained with Annexin V–FITC and PI and analyzed by flow cytometry; the percentage values represent the mean of three independent experiments. B) Western blot analysis of caspase 3 and cleaved caspase 3 in Jurkat cell extracts after 24 h from irradiation (2.5 J/cm^2) in the absence or in the presence of 1 μM compounds. IC = irradiated control.

Compounds **9a-h** were obtained in good yield by direct introduction of the enamino functionality from the corresponding ketones **8a-h**, with *N,N*-dimethylformamide dimethyl acetal (DMFDMA), used as solvent, under conventional heating (70–90%, Method A) or, alternatively, under microwave irradiation (PW 150 W, T 150 °C, 30–45 min) using DMFDMA in a 1:10 ratio and *N,N*-dimethylformamide (DMF) as solvent (95–98%, Method B). At first, the annelation of the pyridine ring was achieved by intermolecular cyclization of the enaminoketones **9** using phenylsulfonylacetonitrile as 1,3-dinucleophile.

This choice was dictated by our previous investigations on pyrroloquinolinones which demonstrated the importance of the phenylsulfonyl group in position 3 of the pyridone moiety in modulating the photoantiproliferative activity. Thus, upon reaction of **9a-h** with phenylsulfonylacetonitrile in refluxing ethanol the desired tricyclic compounds **10a-h** were isolated in good yield (60–88%). Preliminary phototoxicity studies indicated pyrazoloquinolinones **10g,h**, as the most promising and thus selected as lead compounds, for further modifications. Thus, their precursor enaminoketones **9g,h** were further reacted with ethyl cyanoacetate. Reactions, conducted in refluxing ethanol under nitrogen atmosphere, led to desired tricyclic compounds **10i,j** in good yield (72, 80%). Furthermore, for a better evaluation of the structure–activity relationship, the pyridine ring was further functionalized taking advantage of keto–enol tautomerism of the amidic functionality, and subjected to alkylation. Reactions were performed in DMF using NaH as the base and alkyl or arylalkyl halides such as iodomethane and benzyl bromide, allowing the isolation of the corresponding *N*-substituted (**11b-l**, 28–75%) and *O*-substituted (**12b-l**, 22–64%) derivatives from the same reaction mixtures. Tricyclic derivatives **10g,h**, **11g,h** and **12g,h** bearing a 4-chlorophenyl and a 4-methoxyphenyl functionality, selected

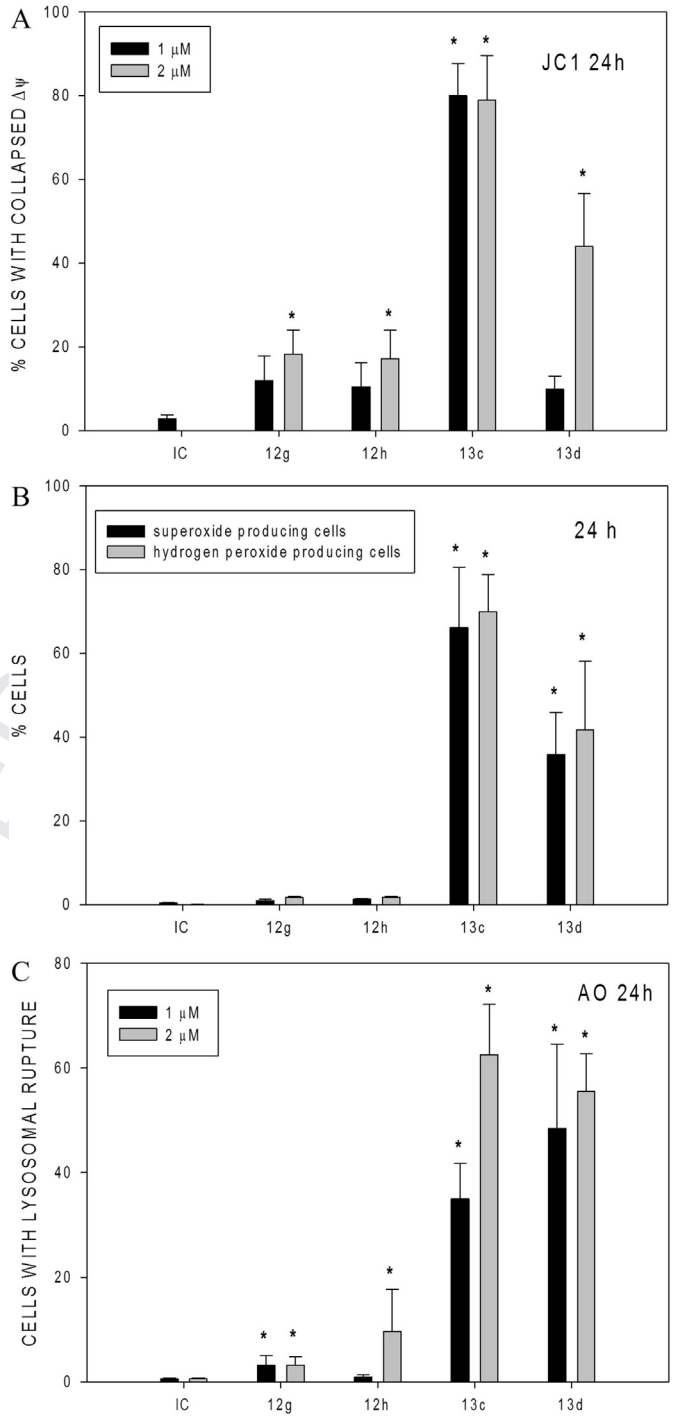

Fig. 4. Mitochondrial (A and B) and lysosomal (C) involvement in photo-induced cell death. A) Percentage of cells with loss of mitochondrial membrane potential (ΔΨ) measured by JC-1 staining 24 h after irradiation (2.5 J/cm^2) of Jurkat cells treated with compounds at 1 or 2 μM; cells were stained with JC-1 and analyzed by fluorescence-activated cell sorting (FACS). B) Production of ROS in Jurkat cells 24 h after irradiation (2.5 J/cm^2) and treatment with compounds at 1 or 2 μM; cells were stained with HE or DCFDA and analyzed by FACS. C) Percentages of cells stained with AO were analyzed by flow cytometry 24 h after irradiation (2.5 J/cm^2) in the presence of compounds at 1 or 2 μM. Values represent the mean ± SEM of three independent experiments. IC = irradiated cells without compound.

according to preliminary data on phototoxicity, were subjected to oxidation with DDQ in dioxane under reflux, allowing the isolation of the corresponding fully aromatic derivatives **13a-f** with yields from moderate to excellent (50–95%).

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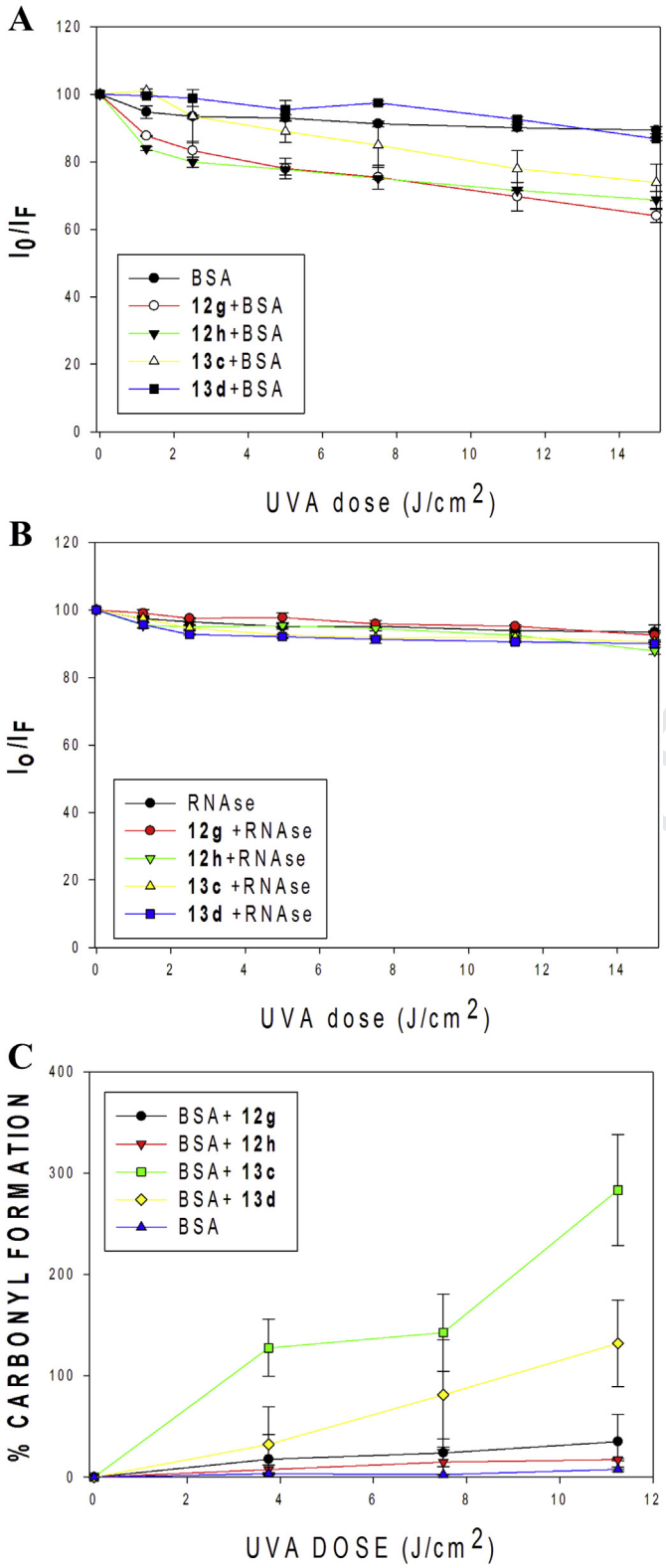

Fig. 5. Photosensitized protein modification by pyrazoloquinolines. BSA and RNAse solutions were irradiated at different UV-A doses in the presence or in the absence of compounds (10 μM). Protein photomodification was evaluated fluorimetrically by monitoring the BSA Trp fluorescence (A) or RNAse Tyr fluorescence (B) or spectrophotometrically by monitoring the carbonyl content in BSA after derivatization with 2,4-dinitrophenylhydrazine (DNPH) (C).

3. Results and discussion

3.1. Spectrophotometric properties

The essential requirement for a photosensitizating compound is the absorption of UV–vis light. All compounds absorbed in the UV–vis range with maximum peaks in the UV-A or in the visible light (338–406 nm) (see Table 1S in the Supporting Materials).

All compounds presented a higher molar extinction coefficient and a batochromic shift in comparison to angelicin (λ_{max} = 305 nm). These effects are linked to the presence of high electron density groups, such as phenylsulphonyl group in position 3 or phenyl group in position 7. Moreover, all compounds were fluorescent.

3.2. Antiproliferative assays

The phototoxicity assays were carried out in some human tumor cell lines by MTT test after 72 h from UV-A irradiation in the presence of the compounds [44]. Moreover, antiproliferative experiments were performed without irradiation by incubating cells with compounds for 72 h to assess a possible cytotoxicity [35]. Results of cytotoxic experiments were shown in Table 2S of Supporting Material, omitting compounds with GI_{50} > 20 μM in all cell lines. Some compounds reduced cellular viability even without irradiation (GI_{50} 16.19–1.46 μM) in some cell lines. However, the cytotoxicity of this class of compounds was very fragmentary and no compounds was active in more than two cell lines. Data of the phototoxicity experiments were reported in Table 2; compounds with GI_{50} > 20 μM in all cell lines were omitted.

Among the dihydro derivatives, compounds **12b-l** having a 2-methoxy group were generally more phototoxic (at the highest UV-A dose: GI_{50} 12.68–0.43 μM) than those bearing the carbonyl group in the same position, **11e-i,k,l** (GI_{50} 13.06–2.44 μM). The only exceptions were **12b**, selectively active in leukemic cell lines, and **12k**, which was devoid of activity.

Among the 2-methoxy substituted quinoline structures, the best results were achieved for derivative **12g** (GI_{50} 1.69–0.43 μM) and **12h** (GI_{50} 1.52–0.53 μM) bearing a 4-chlorophenyl group and a 4-methoxyphenyl group respectively at the pyrazole moiety. Compounds **12g** and **12h** showed an antiproliferative effect at submicromolar level at the highest UV-A dose. It is noticeable that these compounds were more potent than the reference drug angelicin against 3 out of 5 cell lines. Within the series of amidic compounds **10** and **11**, the NH derivatives **10a-j** showed poor activity, with the exception of **10e**, which showed micromolar GI_{50} values on all tested cell lines at the highest dose UV-A (GI_{50} 10.4–2.76 μM). The N-methyl substitution led to an increase of activity, even if compounds bearing the same decoration at the pyrazole moiety were generally less potent than their analogs of the 2-methoxy series (compare **12g** with **11g**, and **12h** with **11h**) with decreased activity either in leukemic cell lines Jurkat and HL-60 and in solid tumors MCF-7, A-549 and LoVo. The presence of a very hydrophobic group in the 7 position seemed to improve the phototoxicity. In fact, compounds bearing an idrogen or a methyl group in the pyrazole nitrogen were inactive, whereas compounds with a phenyl, a benzyl, a chlorophenyl or methoxyphenyl showed a more significative decrease in cell viability.

For the 3-ethoxycarbonyl pyrazolo[3,4-*h*]quinolines, a decrease in the phototoxicity was observed in comparison with the 3-phenylsulfonyl series: compare **12g** (GI_{50} 1.69–0.43 μM) and **12h** (GI_{50} 1.52–0.53 μM) with the corresponding **12i** (GI_{50} 2.30–1.13 μM) and **12j** (GI_{50} 6.43–4.30 μM), inactive on the adenocarcinoma cell line MCF-7.

Among the fully aromatic derivatives **13a-f**, different

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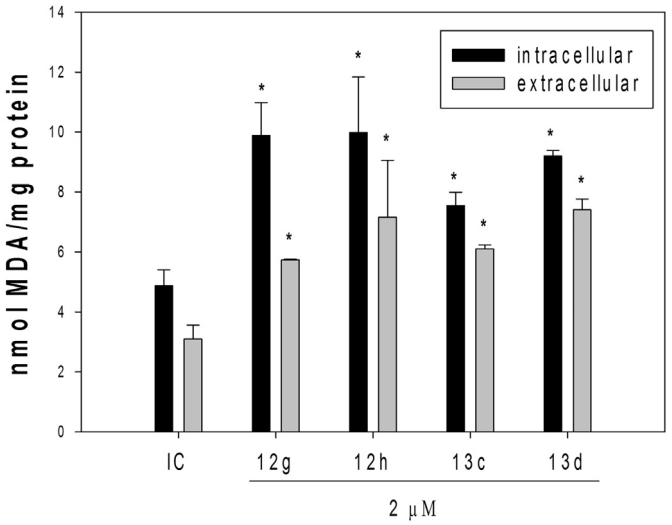


Fig. 6. Lipid peroxidation induced in Jurkat cells 24 h after irradiation (2.5 J/cm^2) in the presence of compounds at 2 μM. The TBA test was performed on the supernatant medium and in the intracellular lisates. Values represent the mean ± SEM of three independent experiments. IC = irradiated cells without compound.

phototoxicity trends were observed.

In fact, the 2-methoxy pyrazolo[3,4-*h*]quinoline **13e,f**, analogs of the most active compounds among the dihydro derivative **12g,h**, maintained a remarkable phototoxicity: they reached the nanomolar level, with range of GI_{50} values of 3.23–0.71 μM for **13e**, and of 0.76–0.09 μM for **13f**, respectively.

The amidic derivatives **13a,b**, were inactive in analogy to the dihydro derivatives **10g,h** after irradiation. Once again, the introduction of a methyl group in the amidic nitrogen led to an increase of phototoxicity: in fact compounds **13c** and **13d** had potent photoantiproliferative effect with GI_{50} values from submicromolar to nanomolar level (0.31–0.04 μM for **13c** and 0.44–0.08 μM for **13d**), demonstrating a remarkable increase of growth inhibitory effect if compared with their dihydro analogs **11g,h**.

Thus, 7-(4-chlorophenyl)-1-methyl-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13c**) and 7-(4-methoxyphenyl)-1-methyl-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13d**) were the most potent compounds of the entire series, with inhibitory capacity on tumor growth in some cases of an order of magnitude higher than that of angelicin. In particular, considering the MCF-7 and LoVo cell lines at the UV-A dose of 3.75 J/cm^2, **13c** was 8 and 27 times, respectively, more potent than angelicin as well as **13d** which was 10 and 12 times more potent.

3.2.1. Colony formation assay

The survival ability of A-549 cells after irradiation with selected pyrazoloquinolinones (**12g**, **12h**, **13c** and **13d**, which were chosen among the most active compounds) was evaluated by colony formation assay [6]. Cells were irradiated and cellular medium was periodically changed until colony formation was assessed by optical microscopy. Colony formation was clearly inhibited by all compounds in a concentration-dependent manner (Fig. 1). The level of colony inhibition was correlated with the phototoxicity data of the previous experiments: in fact, **13c** and **13d** were able to cause a reduction in colony growth at lower concentrations than **12g** and **12h**.

3.2.2. Effect of antioxidant compounds

With the purpose of evaluating which reactive species were involved in the mechanism(s) of phototoxicity, experiments irradiating Jurkat cells with **12g**, **12h**, **13c** and **13d** in the presence of different scavengers were performed, as previously reported [57]. We used the free radical scavengers tocopherol acetate (vitamin E) and glutathione (GSH), the singlet oxygen scavengers sodium azide (NaN_3) and 1,4-diazabicyclo[2.2.2]octane (DABCO), and the hydroxyl radical scavengers dimethylthiourea (DMTU) and mannitol (MANN).

α-Tocopherol was successful in protecting cells from pyrazoloquinolines injuries (Fig. 2A); since vitamin E is a lipophilic vitamin which can protect membranes, we can hypothesize the involvement of the plasma membrane structure in the mechanism of action of these compounds.

The other scavengers exerted their action in a different manner for each molecule: for example, for **12g** and **12h**, the lipophylic scavangers increased the cellular viability, while all scavangers led to an increase of cellular survival in the presence of **13c**. The phototoxicity of **12h** was mainly reduced in the presence of

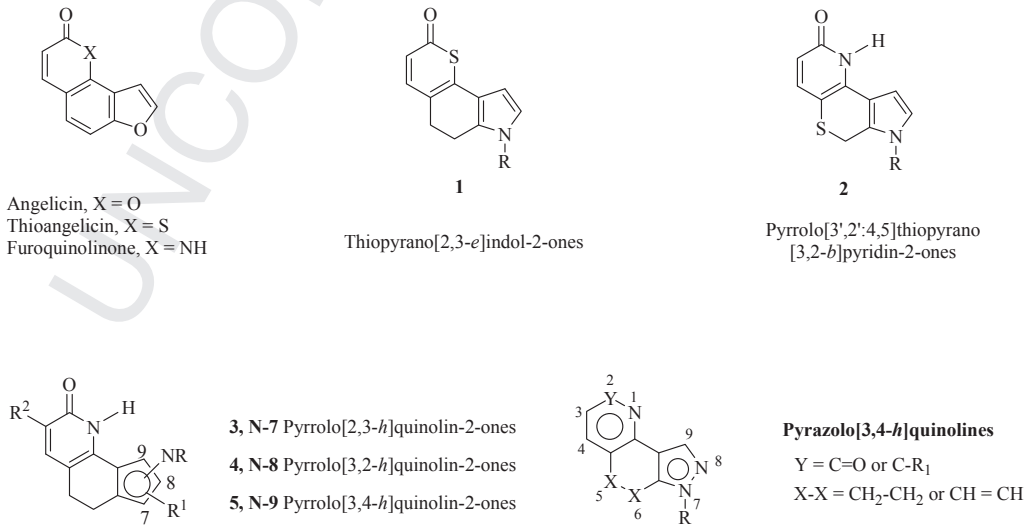
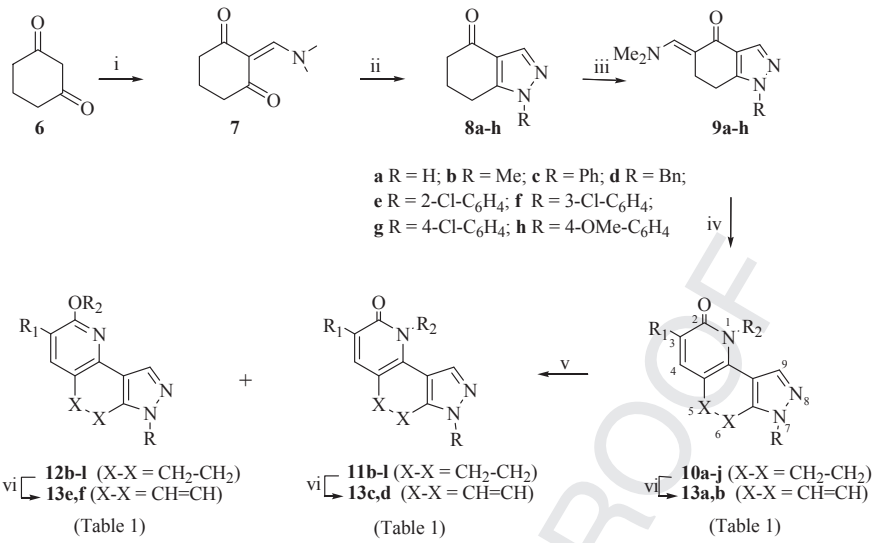


Chart 1. Structures of angelicin, thioangelicin, furoquinolinone; thiopyrano[2,3-*e*]indol-2-ones (**1**); pyrrolo[3′,2′:4,5]thiopyrano[3,2-*b*]pyridin-2-ones (**2**); pyrrolo[2,3-*h*]quinolin-2-ones (**3**); pyrrolo[3,2-*h*]quinolin-2-ones (**4**); pyrrolo[3,4-*h*]quinolin-2-ones (**5**); pyrazolo[3,4-*h*]quinolines.



Scheme 1. *Reagents and conditions*: (i) DMFDMA, 96%; (ii) H_2NNHR, ethanol/acetic acid (2:1) or glacial acetic acid, reflux, 2 h, 60–93%; (iii) DMFDMA, reflux, 24 h, 70–90% (Method A), or DMFDMA, MW (PW 150 W, T 150 °C), 30–45 min, 95–98% (Method B); (iv) $PhSO_2CH_2CN$ or $NCCH_2COOEt$, ethanol, reflux, 24 h, 60–88%; (v) NaH, DMF, rt, 3 h then MeI or BnBr rt, 24 h, 22–75%; (vi) for **10g,h**, **11g,h** and **12g,h**: DDQ, dioxane, reflux, 24 h, 50–95%.

Table 1
Pyrazolo[3,4-*h*]quinolines **10**–**13**.

Cpd	R	R^1	R^2	X–X	Sbt	Yld(%)
10a	H	SO_2Ph	H	CH_2–CH_2	**9a**	60
					8	40
10b	Me	SO_2Ph	H	CH_2–CH_2	**9b**	80
10c	Ph	SO_2Ph	H	CH_2–CH_2	**9c**	63
10d	Bn	SO_2Ph	H	CH_2–CH_2	**9d**	88
10e	2-Cl–C_6H_4	SO_2Ph	H	CH_2–CH_2	**9e**	65
						12
10f	3-Cl–C_6H_4	SO_2Ph	H	CH_2–CH_2	**9f**	68
10g	4-Cl–C_6H_4	SO_2Ph	H	CH_2–CH_2	**9g**	65
10h	4-OMe–C_6H_4	SO_2Ph	H	CH_2–CH_2	**9h**	73
10i	4-Cl–C_6H_4	CO_2Et	H	CH_2–CH_2	**9g**	72
10j	4-OMe–C_6H_4	CO_2Et	H	CH_2–CH_2	**9h**	80
11b	Me	SO_2Ph	Me	CH_2–CH_2	**10b**	58
11c	Ph	SO_2Ph	Me	CH_2–CH_2	**10c**	58
11d	Bn	SO_2Ph	Me	CH_2–CH_2	**10d**	68
11e	2-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10e**	58
11f	3-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10f**	56
						15
11g	4-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10g**	53
11h	4-OMe–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10h**	59
11i	4-Cl–C_6H_4	CO_2Et	Me	CH_2–CH_2	**10i**	70
11j	4-OMe–C_6H_4	CO_2Et	Me	CH_2–CH_2	**10j**	60
11k	4-Cl–C_6H_4	SO_2Ph	Bn	CH_2–CH_2	**10g**	30
11l	4-OMe–C_6H_4	SO_2Ph	Bn	CH_2–CH_2	**10h**	28
12b	Me	SO_2Ph	Me	CH_2–CH_2	**10b**	35
12c	Ph	SO_2Ph	Me	CH_2–CH_2	**10c**	30
12d	Bn	SO_2Ph	Me	CH_2–CH_2	**10d**	30
12e	2-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10e**	33
12f	3-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10f**	35
12g	4-Cl–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10g**	30
12h	4-OMe–C_6H_4	SO_2Ph	Me	CH_2–CH_2	**10h**	36
12i	4-Cl–C_6H_4	CO_2Et	Me	CH_2–CH_2	**10i**	22
12j	4-OMe–C_6H_4	CO_2Et	Me	CH_2–CH_2	**10j**	35
12k	4-Cl–C_6H_4	SO_2Ph	Bn	CH_2–CH_2	**10g**	64
12l	4-OMe–C_6H_4	SO_2Ph	Bn	CH_2–CH_2	**10h**	55
13a	4-Cl–C_6H_4	SO_2Ph	H	CH=CH	**10g**	50
13b	4-OMe–C_6H_4	SO_2Ph	H	CH=CH	**10h**	60
13c	4-Cl–C_6H_4	SO_2Ph	Me	CH=CH	**11g**	93
13d	4-OMe–C_6H_4	SO_2Ph	Me	CH=CH	**11h**	95
13e	4-Cl–C_6H_4	SO_2Ph	Me	CH=CH	**12g**	65
13f	4-OMe–C_6H_4	SO_2Ph	Me	CH=CH	**12h**	86

scavengers for free or hydroxyl scavangers like many other photochemotherapics synthesized by some of us [44,46]. For the other compounds, singlet oxygen could be implicated in their phototoxicity since NaN_3 or DABCO showed a protective effect.

Anyway, the most effective scavengers have often lipophilic features, so they probably have the same localization of pyrazoloquinolines.

3.3. Determination of reactive oxygen species

Production of reactive oxygen species is one of the mechanisms underlying drug induced photosensitization damage. Since some scavengers led to an increase in cell viability, the determination of some oxygen reactive species, such as singlet oxygen and superoxide anion, was assessed *in vitro* in aqueous solution after UV-A irradiation as described in Ref. [58]. Superoxide formation can be linked to type I photosensization mechanism, which involves free radical intermediates and ground state oxygen; while formation of singlet oxygen by energy transfer can be observed in type II [59]. Compounds **13c** and **13d** efficiently generated singlet oxygen (Fig. 2B; RNO bleaching) and superoxide anion (Fig. 2C; increase in absorbance at 560 nm) under UV-A irradiation, while the production of ROS of the other two compounds was very low. In particular, **13c** and **13d** generated comparable levels of superoxide anion and higher levels of singlet oxygen than the angelicin reference compound [60], suggesting an involvement of both oxygen-dependent photosensization mechanisms.

3.4. Cell death evaluation

To gain a better understanding of the cell death mechanisms that underlined the antiproliferative activities of this series of compounds, we performed some flow cytofluorimetric and immunoblotting experiments with some of the most active compounds: **12g**, **12h**, **13c** and **13d**. Cell death by apoptosis is desirable for a chemotherapic drug, since the apoptotic process does not cause inflammatory reaction as there is no release of intracellular components like in the necrotic death [60]. First, the typical apoptotic loss of plasmatic membrane asymmetry was evaluated by

Table 2
Photocytotoxicity GI_{50} (μM)[a] of pyrazolo[3,4-*h*]quinolines **10**–**13** in human tumor cell lines after 72 h.

	JURKAT		HL-60		MCF-7		A-549		LoVo	
	2.5 J/cm^2	1.25 J/cm^2	2.5 J/cm^2	1.25 J/cm^2	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2	2.5 J/cm^2
Ang	1.00 ± 0.20[b]	n.d.	1.20 ± 0.10	n.d.	1.50 ± 0.2	4.42 ± 0.53	n.d.	n.d.	1.11 ± 0.41	4.00 ± 0.40
10c	>20	>20	>20	>20	>20	>20	14.06 ± 0.94	>20	>20	>20
10d	6.88 ± 0.94	>20	5.06 ± 0.63	>20	>20	>20	>20	>20	14.52 ± 2.43	>20
10e	3.95 ± 0.34	11.27 ± 1.46	2.76 ± 0.40	11.48 ± 1.69	10.4 ± 0.60	12.99 ± 1.48	8.97 ± 0.95	13.33 ± 2.20	10.14 ± 1.09	>20
10f	>20	>20	>20	>20	>20	>20	9.53 ± 1.18	14.14 ± 1.51	>20	>20
10i	>20	>20	>20	>20	9.85 ± 0.93	7.32 ± 0.98	>20	>20	>20	>20
11c	6.99 ± 1.15	>20	>20	>20	>20	>20	>20	>20	>20	>20
11d	5.67 ± 0.93	>20	6.79 ± 0.54	>20	>20	>20	>20	>20	13.04 ± 2.20	>20
11e	5.21 ± 0.84	>20	4.82 ± 0.24	>20	12.62 ± 1.04	>20	13.02 ± 1.27	>20	13.06 ± 1.85	>20
11f	2.81 ± 0.41	>20	2.44 ± 0.24	>20	9.01 ± 1.19	12.67 ± 1.74	8.40 ± 1.43	>20	7.54 ± 1.17	13.12 ± 1.05
11g	3.01 ± 0.60	>20	2.88 ± 0.42	>20	>20	>20	>20	>20	11.11 ± 2.40	>20
11h	6.68 ± 0.42	>20	7.65 ± 0.55	>20	>20	>20	>20	>20	>20	>20
11i	>20	>20	7.67 ± 1.10	>20	>20	>20	>20	>20	>20	>20
11k	4.97 ± 1.45	>20	7.17 ± 1.44	>20	9.89 ± 1.45	>20	6.64 ± 1.12	>20	3.72 ± 0.91	11.08 ± 1.24
11l	>20	>20	>20	>20	>20	>20	>20	>20	4.12 ± 1.19	>20
12b	12.68 ± 1.58	>20	9.79 ± 1.46	>20	>20	>20	>20	>20	>20	>20
12c	0.71 ± 0.13	3.65 ± 0.47	0.79 ± 0.06	2.83 ± 0.31	1.84 ± 0.19	2.47 ± 0.34	2.89 ± 0.32	7.56 ± 0.69	1.10 ± 0.20	3.14 ± 0.30
12d	1.40 ± 0.15	2.48 ± 0.48	1.30 ± 0.16	3.83 ± 0.57	3.05 ± 0.48	4.00 ± 0.51	5.74 ± 1.04	10.37 ± 1.48	1.41 ± 0.25	5.32 ± 0.30
12e	0.90 ± 0.13	1.93 ± 0.14	0.81 ± 0.15	3.78 ± 0.53	1.59 ± 0.12	3.13 ± 0.35	4.80 ± 0.50	14.32 ± 2.66	1.78 ± 0.36	5.73 ± 0.51
12f	1.34 ± 0.27	1.95 ± 0.35	0.53 ± 0.14	3.42 ± 0.46	2.52 ± 0.40	5.15 ± 1.10	6.61 ± 0.85	13.33 ± 3.48	2.99 ± 0.87	9.84 ± 1.14
12g	0.43 ± 0.13	0.72 ± 0.11	0.48 ± 0.10	4.03 ± 1.19	1.69 ± 0.18	7.09 ± 2.02	1.49 ± 0.35	5.28 ± 1.03	0.73 ± 0.15	5.53 ± 1.00
12h	0.64 ± 0.15	1.15 ± 0.18	0.72 ± 0.12	5.29 ± 1.21	1.18 ± 0.12	2.53 ± 0.24	1.52 ± 0.15	4.02 ± 0.44	0.53 ± 0.11	3.56 ± 0.63
12i	1.81 ± 0.35	4.47 ± 0.72	1.71 ± 0.27	5.38 ± 1.01	>20	>20	2.30 ± 0.31	3.68 ± 0.61	1.13 ± 0.41	2.79 ± 0.47
12j	6.43 ± 0.55	9.93 ± 1.31	4.69 ± 0.67	7.71 ± 1.41	>20	>20	4.67 ± 0.56	6.99 ± 0.74	4.30 ± 0.52	5.81 ± 0.32
12l	>20	>20	9.09 ± 1.38	>20	>20	>20	5.74 ± 0.96	>20	2.76 ± 0.84	>20
13b	>20	>20	>20	>20	>20	>20	4.26 ± 0.67	5.39 ± 0.93	>20	>20
13c	0.10 ± 0.02	0.20 ± 0.06	0.31 ± 0.02	0.39 ± 0.05	0.17 ± 0.03	0.33 ± 0.06	0.08 ± 0.03	0.26 ± 0.25	0.04 ± 0.02	0.16 ± 0.03
13d	0.25 ± 0.04	0.58 ± 0.15	0.44 ± 0.03	0.61 ± 0.08	0.14 ± 0.03	0.29 ± 0.03	0.08 ± 0.03	0.27 ± 0.01	0.09 ± 0.02	0.19 ± 0.03
13e	0.96 ± 0.20	4.23 ± 1.15	1.20 ± 0.14	2.16 ± 0.24	3.23 ± 0.46	5.36 ± 0.73	0.75 ± 0.21	3.09 ± 1.15	0.71 ± 0.15	1.43 ± 0.30
13f	0.54 ± 0.11	1.50 ± 0.40	0.76 ± 0.12	0.99 ± 0.07	0.59 ± 0.07	1.23 ± 0.14	0.09 ± 0.03	0.35 ± 0.05	0.16 ± 0.03	0.42 ± 0.02

[a] GI_{50} = the concentration that causes the 50% growth inhibition.
[b] Data are expressed as mean ± S.E.M. of at least four different independent experiments.

flow cytometry. In this cytofluorimetric analysis, two fluorophores were used 24 h after irradiation of Jurkat cells with the selected compounds: PI (propidium iodide), which stains DNA and is permeable only to dead cells, and fluorescent immunolabeling of the protein Annexin-V-FITC, which binds to phosphatidylserine (PS). PS is a phospholipid that flips from the inner to the outer leaflet of the plasma membrane during apoptosis. Positive staining with Annexin-V correlates with the loss of plasma membrane asymmetry but precedes the complete loss of membrane integrity that accompanies the later stages of *in vitro* cell death. In contrast, PI can only enter cells after the loss of their membrane integrity. Thus, dual staining with Annexin-V and PI allows clearly to discriminate between unaffected cells (Annexin-V−/PI−), early apoptotic cells (Annexin-V+/PI−), late apoptotic cells (Annexin-V+/PI+), and necrotic cells (Annexin-V−/PI+) [61].

After 24 h from irradiation, all compounds were able to cause an important drop in alive cells in comparison to irradiated control and a clear increase in Annexin-V+/PI + cells (Fig. 3A).

Caspases activation, which occurs at relatively early stages of apoptosis, is also considered a hallmark of this mode of cell death [62]; thus, an immunoblotting test was carried out to verify a possible activation of caspase 3 by compounds. Caspase 3 is activated after the cleavage of active portion of pro-caspase 3 by other caspases. The levels of cleaved caspase 3 were evaluated by immunoblotting in cellular lisates after 24 h from irradiation. The level of cleaved caspase was very low when irradiation was carried out in the presence of **12g** and **12h** while it was much higher in the case of compounds **13c** and **13d** (Fig. 3B). Our hypothesis was that **12g** and **12h** induced cell death mainly by necrosis while **13c** and **13d** mainly by apoptosis.

3.4.1. Mitocondrial and lysosomal integrity assessment

To better understand the mode of photoinduced cellular death by pyrazoloquinolines, some flow cytometric tests which assessed the involvement of some intracellular organelles were performed. The complex role of mitochondria in apoptosis was identified by numerous biochemical studies that pointed out that many mitochondrial proteins are able to directly activate cellular apoptotic programs [63]. Two different tests were carried out to check mitochondrial dysfunction: variations in mitochondrial membrane potential ($\Delta\Psi_M$) and reactive oxygen species (ROS) generation. Mitochondrial membrane depolarization and the decrease of mitochondrial potential can be assessed by flow cytometry using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzylimidazolil-carbocianine chloride) as a probe. JC-1 is a lypophylic fluorescent cation that is able to enter mitochondria and to change its fluorescent properties according to mitochodrial potential. In fact, it forms orange fluorescence aggregates with normal mitochondrial potential, whereas when the mitochondrial membrane is depolarized (low $\Delta\Psi_M$), JC-1 forms monomers which emit at 530 nm [64]. For this test, Jurkat cells were irradiated (2.5 J/cm^2) in the presence of 1 or 2 μM **12g, 12h, 13c** and **13d**. After 24 h from irradiation, mitochondrial depolarization occurred for all compounds in different extents, however, **13c** was able to photoinduce a higher drop in mitochondrial potential (Fig. 4A).

The reduction of $\Delta\Psi_M$, concomitant with uncoupling of mitochondrial electron chain transport and ATP synthesis, causes increased generation of ROS, which, in turn, are important mediators of apoptosis [65]. The ROS production was assessed by flow cytometry after 24 h from irradiation of Jurkat cells in the presence of the tested compounds. Two probes were used: one for the

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detection of superoxide anion and the other for the detection of hydrogen peroxide [66]. A clear increase in ROS production was observed when Jurkat cells were irradiated in the presence of **13c** and **13d**, while no significant increase was assessed with **12g** and **12h**. In conclusion, mitochondria were deeply involved only in cell death photoinduced by **13c** and **13d** (Fig. 4B).

Other cellular organelles such as lysosomes can be involved in the ordered propagation of apoptotic events [67]. To evaluate the involvement of lysosomes in cell death induction, the acridine orange (AO) re-uptake cytofluorimetric test was carried out as previously reported [68]. Cells irradiated in the presence of **12g** and **12h** did not show an important involvement of lysosomes in cell death mechanism, while lysosomes were clearly damaged after irradiation in the presence of **13c** and **13d** (Fig. 4C).

3.5. DNA binding and photodamage

Since DNA is the major target of furocoumarins for their photocytotoxicity [69], the binding of the test compounds to this macromolecule was investigated. UV–vis and fluorimetric titration with DNA did not show any variation of the absorption spectra or the presence of isosbestic points indicative of the formation of a complex between the test compounds and the DNA. Furthermore, a poor fluorescence quenching of the emission of the free drug in the presence of DNA suggested that the title compounds were loosely bound to DNA (data not shown). Even if the binding to DNA was very low, a possible photodamage to this macromolecule was also investigated. In particular, the photoinduction of frank strand breaks was evaluated irradiating a supercoiled double stranded plasmid (pBR322) in the presence of the tested compounds. The formation of open circular (OC) or linear (L) form from supercoiled (SC) DNA was monitored by separating them by agarose electrophoresis. Moreover, oxidative damages were assessed incubating the irradiated mixtures (pBR322 + tested compounds) with repair enzymes such as Fpg and Endo III, which recognized purine and pirimidine damages, respectively [70,71]. For this test, solutions of pBR322 were irradiated (UV-A dose: 7.5 J/cm^2) in the presence or absence of compounds **12g**, **12h**, **13c** and **13d** in rate [compound]/[DNA] = 3/1 (See Fig. 1S).

Gel analysis showed that compounds were not able to induce frank strand breaks after UV-A irradiation; moreover, no oxidative damages were assessed in DNA bases. All together these data suggested that DNA was not the target of photodamage of these compounds.

3.6. Protein photodamage

A possible protein photodamage was also evaluated since these macromolecules represent the 68% of cellular dry weight and may be an important target for various photosensitizing agents [72]. Moreover, proteins present endogen chromophores within their structure, such as aromatic aminoacids or prosthetic groups (flavins and heme), or may bind exogenous chromophores which can be easily assessed for possible photoinduced damages or changes. Proteins can easily react with ROS which can induce photooxidative damage causing for example, loss in activity, fragmentation, aggregation, denaturation, modifications in hydrophobicity or structure [73]. In order to investigate if proteins were photodamaged by the tested compounds, solutions containing bovine serum albumin (BSA) or ribonuclease A (RNAseA), as models, and pyrazoloquinolines **12g**, **12h**, **13c** and **13d** were irradiated several times. The protein photodamage was first assessed by monitoring the protein fluorescence due to tryptophan (Trp) in BSA sample and tyrosine (Tyr) in RNAse one. Then, the degree of oxidative modifications was measured by monitoring the carbonyl content. Results were reported in Fig. 5 and demonstrated that the amount of the aromatic aminoacid Trp was reduced in UV-A dependent mode for all compounds with the exception of **13d** (Fig. 5A). No significant decrease in Tyr fluorescence was observed if RNAase was irradiated in the presence of the tested compounds (Fig. 5B). The carbonyl content in BSA increased for all compounds after UV-A irradiation; however, **13c** and **13d** were able to photoinduce higher carbonyl formation rate. Pyrazoloquinazolines caused an oxidative damage in model proteins even if not all the aminoacids could be involved and these macromolecules could be an intracellular target of these compounds.

3.7. Lipid peroxidation

Pyrazoloquinolines are hydrophobic molecules, thus they probably localize in hydrophobic cellular structures such as cellular membranes, which could be easy target of photodamage induced by the tested compounds. The thiobarbituric acid (TBA) assay was used to determine whether lipid peroxidation occurred upon irradiation of Jurkat cells incubated in the presence of test compounds. TBA reacts with one of the final product of lipid peroxidation, such as malondialdehyde [74]. The TBA test was performed on the supernatant medium and in the cell homogenates 24 h after irradiation. Thiobarbituric reactive substances (TBARS) were significantly produced when the cells were exposed to the compounds and UV-A both in the supernatant and in the cell extract (Fig. 6). Thus, lipids could be an important target of compound photodamage.

4. Conclusion

A new series of pyrazolo[3,4-*h*]quinolines has been successfully prepared by annelation of the pyridine ring on the indazole moiety. The synthetic pathway presented herein was never described before in the pyrazole series and proved to be versatile allowing the preparation of a large number of derivatives. Some of them showed potent inhibitory growth effect under UV light irradiation, being from 8 to 27 times more active than the reference drug. Evaluation of SAR of a number of compounds indicated that the presence of 4-chlorophenyl or a 4-methoxyphenyl substituent in the pyrazolo [3,4-*h*]quinolines structure is crucial to express the best activity.

We deeply analyzed the photodamage induced by four among the most phototoxic compounds: in particular, two of them, **12g** and **12h** were dihydro derivatives while **13c** and **13d** presented the lowest GI_{50} values and were fully aromatic molecules. They showed some differences in their mode of inducing cellular death: in fact, the dihydro derivatives mainly caused necrosis with a minimal involvement of mitochondria and lysosomes, whereas **13c** and **13d** were associated with apoptosis with a large mitochondrial and lysosomal involvement. Interestingly, these latter generated comparable levels of superoxide anion and higher levels of singlet oxygen than the angelicin reference compound, suggesting an involvement of both oxygen-dependent photosensization mechanisms (type I and type II).

We hypothesized that lipids were the main target of pyrazoloquinoline photodamage as these compounds were able to induce high degrees of lipid peroxidation and their phototoxicity was really decreased when cellular irradiation was carried in the presence of vitamin E. Interestingly, DNA seemed not to be involved in pyrazoloquinoline photodamage which is crucial and a clear advantage, possibly preventing some typical side effects of furocumarins such as cancerogenesis and mutagenesis.

In conclusion, compounds **12g**, **12h**, **13c** and **13d** can be recognized as the lead compounds of the class of pyrazolo[3,4-*h*]quinolines and could represent the seeds for further developments directed to the improvement of water solubility. Moreover, the

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design of new bathochromically-shifted derivatives in their absorption spectrum which could represent a very appealing target. This class of compounds have been already covered by a patent [75] and a clinical development is desirable.

5. Experimental

5.1. Synthesis and characterization

All melting points were taken on a Buchi-Tottoli capillary apparatus; IR spectra were determined, in $CHBr_3$, with a Jasco FT/IR 5300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured in DMSO-d_6 or $CDCl_3$ solutions, at 200 and 50 MHz respectively, using a Bruker Avance II series 200 MHz spectrometer. Column chromatography was performed with Merck silica gel 230–400 Mesh ASTM or with a SEPACOR chromatography apparatus BÜCHI. Elemental analyses (C, H, N) were within ±0.4% of the theoretical values.

Compound **7** was prepared according to procedure described elsewhere [39].

5.1.1. General procedure for the preparation of 1-substituted 1,5,6,7-tetrahydro-4H-indazol-4-ones(**8a-h**)

To a solution of compound **7** (3 g, 18 mmol) in a mixture of ethanol and acetic acid (2:1) (48 mL) or, for the synthesis of compounds **8 e-g**, only glacial acetic acid (30 mL) the suitable hydrazine (18 mmol) was added and the reaction mixture was heated under reflux for 2 h. Then, the reaction mixture was poured onto crushed ice and the solid was filtered off, dried and purified by chromatography (DCM/AcOEt 9:1) as eluent.

*5.1.1.1. 1,5,6,7-Tetrahydro-4H-indazol-4-one (**8a**).* This compound was obtained from reaction of **7** with hydrazine hydrate. Yellow solid; yield: 67%; m.p. 165–166 °C; IR cm^{-1}: 3186 (NH), 1647 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 1.96–2.08 (m, 2H, CH_2), 2.37 (t, J = 6.1 Hz, 2H, CH_2), 2.81 (t, J = 6.1 Hz, 2H, CH_2), 7.91 (s, 1H, H-3), 13.21 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 21.1 (t), 23.3 (t), 38.1 (t), 117.8 (d), 133.8 (s), 155.2 (s), 192.9 (s). Anal calcd for $C_7H_8N_2O$ (136.15): C, 61.75; H, 5.92; N, 20.58. Found: C, 61.88; H, 6.11; N, 20.39.

*5.1.1.2. 1-Methyl-1,5,6,7-tetrahydro-4H-indazol-4-one (**8b**).* This compound was obtained from reaction of **7** with methylhydrazine. Brown solid; yield: 60%; m.p. 95–96 °C; IR cm^{-1}: 1668 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.01–2.07 (m, 2H, CH_2), 2.35 (t, J = 6.2 Hz, 2H, CH_2), 2.84 (t, J = 6.2 Hz, 2H, CH_2), 3.77 (s, 3H, CH_3), 7.74 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.3 (t), 27.7 (t), 35.9 (q), 37.4 (t), 118.5 (s), 135.8 (d), 149.9 (s), 192.1 (s). Anal. $C_8H_{10}N_2O$ (150.18): C, 63.98; H, 6.71 N, 18.65. Found: C, 64.10; H, 6.55; N, 18.80.

*5.1.1.3. 1-Phenyl-1,5,6,7-tetrahydro-4H-indazol-4-one (**8c**).* This compound was obtained from reaction of **7** with phenylhydrazine. Brown solid; yield: 65%; m.p. 137–138 °C; IR cm^{-1}: 1664 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.03–2.13 (m, 2H, CH_2), 2.45 (t, J = 6.1 Hz, 2H, CH_2), 3.01 (t, J = 6.1 Hz, 2H, CH_2), 7.47–7.66 (m, 5H, Ar), 8.05 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.4 (t), 23.1 (t), 37.4 (t), 119.9 (s), 123.4 (2 × d), 128.4 (d), 129.4 (2 × d), 137.7 (d), 138.4 (s), 149.9 (s), 192.6 (s). Anal calcd for $C_{13}H_{12}N_2O$ (212.25): C, 73.56; H, 5.70; N, 13.20. Found: C, 73.68; H, 5.59; N, 13.48.

*5.1.1.4. 1-Benzyl-1,5,6,7-tetrahydro-4H-indazol-4-one (**8d**).* This compound was obtained from reaction of **7** with benzylhydrazine. Brown oil; yield: 63%; IR cm^{-1}: 1653 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 1.97–2.09 (m, 2H, CH_2), 2.36 (t, J = 6.2 Hz, 2H, CH_2), 2.85 (t, J = 6.2 Hz, 2H, CH_2), 5.36 (s, 2H, CH_2), 7.19–7.40 (m, 5H, Ar), 7.82 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.6 (t), 22.8 (t), 37.4 (t), 52.2 (t), 118.8 (s), 127.4 (2 × d), 127.7 (d), 128.6 (2 × d), 136.4 (s), 136.5 (d), 149.9 (s), 192.2 (s). Anal calcd for $C_{14}H_{14}N_2O$ (226.27): C, 74.31; H, 6.24; N, 12.38. Found: C, 74.18; H, 6.36; N, 12.22.

*5.1.1.5. 1-(2-Chlorophenyl)-1,5,6,7-tetrahydro-4H-indazol-4-one (**8e**).* This compound was obtained from reaction of **7** with 2-chlorophenylhydrazine. Pale brown solid; yield: 62%; m.p. 126–127 °C; IR cm^{-1}: 1672 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.03–2.12 (m, 2H, CH_2), 2.45 (t, J = 6.1 Hz, 2H, CH_2), 2.66 (t, J = 6.1 Hz, 2H, CH_2), 7.56–7.79 (m, 4H, Ar), 8.07 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 21.0 (t), 22.9 (t), 37.5 (t), 119.0 (s), 128.5 (d), 129.6 (d), 130.4 (d), 130.4 (s), 131.6 (d), 135.4 (s), 137.7 (d), 151.9 (s), 192.4 (s). Anal calcd for $C_{13}H_{11}ClN_2O$ (246.69): C, 63.29; H, 4.49; N, 11.36. Found: C, 63.37; H, 4.35; N, 11.51.

*5.1.1.6. 1-(3-Chlorophenyl)-1,5,6,7-tetrahydro-4H-indazol-4-one (**8f**).* This compound was obtained from reaction of **7** with 3-chlorophenylhydrazine. Brown solid; yield: 93%; m.p. 92–93 °C; IR cm^{-1}: 1664 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.05–2.11 (m, 2H, CH_2), 2.45 (t, J = 6.2 Hz, 2H, CH_2), 3.05 (t, J = 6.2 Hz, 2H, CH_2), 7.54–7.73 (m, 4H, Ar), 8.07 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.4 (t), 23.0 (t), 37.4 (t), 120.2 (s), 122.0 (d), 123.2 (d), 128.0 (d), 131.2 (d), 133.7 (s), 138.2 (d), 139.6 (s), 150.3 (s), 192.6 (s). Anal calcd for $C_{13}H_{11}ClN_2O$ (246.69): C, 63.29; H, 4.49; N, 11.36. Found: C, 63.41; H, 4.32; N, 11.55.

*5.1.1.7. 1-(4-Chlorophenyl)-1,5,6,7-tetrahydro-4H-indazol-4-one (**8g**).* This compound was obtained from reaction of **7** with 4-chlorophenylhydrazine. Brown solid; yield: 74%; m.p. 133–134 °C; IR cm^{-1}: 1670 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.01–2.13 (m, 2H, CH_2), 2.45 (t, J = 6.1 Hz, 2H, CH_2), 3.02 (t, J = 6.1 Hz, 2H, CH_2), 7.63 (d, J = 9.3 Hz, 2H, H-3″ and H-5″), 7.68 (d, J = 9.3 Hz, 2H, H-2″ and H-6″), 8.07 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.4 (t), 23.0 (t), 37.4 (t), 120.1 (s), 125.0 (2 × d), 129.4 (2 × d), 132.4 (s), 137.2 (d), 138.0 (s), 150.1 (s), 192.6 (s). Anal calcd for $C_{13}H_{11}ClN_2O$ (246.69): C, 63.29; H, 4.49; N, 11.36. Found: C, 63.18; H, 4.63; N, 11.46.

*5.1.1.8. 1-(4-Methoxyphenyl)-1,5,6,7-tetrahydro-4H-indazol-4-one (**8h**).* This compound was obtained from reaction of **7** with 4-methoxyphenylhydrazine. Brown solid; yield: 70%; m.p. 153–154 °C; IR cm^{-1}: 1668 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.02–2.11 (m, 2H, CH_2), 2.43 (t, J = 6.1 Hz, 2H, CH_2), 2.94 (t, J = 6.1 Hz, 2H, CH_2), 3.83 (s, 3H, CH_3), 7.10 (d, J = 9.0 Hz, 2H, H-3″ and H-5″), 7.53 (d, J = 9.0 Hz, 2H, H-2″ and H-6″), 8.01 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.2 (t), 23.0 (t), 37.4 (t), 57.4 (q), 114.4 (2 × d), 119.6 (s), 125.0 (2 × d), 131.4 (d), 137.3 (s), 149.6 (s), 158.8 (s), 192.5 (s). Anal calcd for $C_{14}H_{14}N_2O_2$ (242.27): C, 69.41; H, 5.82; N, 11.56. Found: C, 69.56; H, 5.71; N, 11.39.

5.1.2. General procedure for the preparation of 1-substituted 5-[(dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-ones (**9a-h**)

A solution of the suitable ketone **8a-h** (5.3 mmol) in DMFDMA (8 mL) was heated at reflux for 24 h (Method A). Then the reaction mixture was cooled and the solvent was removed under reduced pressure. The crude was stirred in diethyl ether.

Alternatively to a solution of the suitable ketone **8a-h** (1.5 mmol) in anhydrous DMF (2 mL), DMFDMA (2 mL, 15 mmol) was added and the mixture was stirred under microwave irradiation (Method B). (MW conditions: Power 150 W; Time 30–45 min; Temperature (max) 150 °C). The reaction mixture was poured onto

crushed ice.

Enaminoketones having a solid state, were filtered off, washed with diethyl ether and dried. The oily ones were decanted, solubilized in dichloromethane and the organic layer was dried (Na_2SO_4) and the solvent was removed under reduced pressure.

*5.1.2.1. 5-[(Dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-one (**9a**).* This compound was obtained from **8a**. Brown solid; yield: 90% (Method A), 98% (Method B); m.p. 131–132 °C; IR cm^{-1}: 3116 (NH), 1668 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.31–2.39 (m, 4H, 2 × CH_2), 3.77 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 7.74 (s, 1H, CH), 7.96 (s, 1H, H-3), 13.23 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.2 (t), 23.3 (t), 37.4 (2 × q), 118.4 (s), 130.7 (d), 135.9 (d), 149.9 (s), 156.3 (s), 192.2 (s). Anal calcd for $C_{10}H_{13}N_3O$ (191.23): C, 62.81; H, 6.85; N, 21.97. Found: C, 62.99; H, 6.72; N, 22.06.

*5.1.2.2. 5-[(Dimethylamino)methylidene]-1-methyl-1,5,6,7-tetrahydro-4H-indazol-4-one (**9b**).* This compound was obtained from **8b**. Brown oil; yield: 77% (Method A), 96% (Method B); IR cm^{-1}: 1639 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.74 (t, J = 6.4 Hz, 2H, CH_2), 2.96 (t, J = 6.4 Hz, 2H, CH_2), 3.05 (s, 6H, 2 × CH_3), 3.74 (s, 3H, CH_3), 7.29 (s, 1H, CH), 7.61 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.2 (t), 22.0 (t), 34.6 (q), 42.1 (2 × q), 99.9 (s), 118.5 (s), 135.1 (d), 145.6 (s), 146.5 (d), 180.5 (s). Anal. $C_{11}H_{15}N_3O$ (205.26): C, 64.37; H, 7.37; N, 20.47. Found: C, 64.25; H, 7.51; N, 20.58.

*5.1.2.3. 5-[(Dimethylamino)methylidene]-1-phenyl-1,5,6,7-tetrahydro-4H-indazol-4-one (**9c**).* This compound was obtained from **8c**. Brown oil; yield: 80% (Method A), 98% (Method B); IR cm^{-1}: 1657 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.80–2.95 (s, 4H, 2 × CH_2), 3.07 (s, 6H, 2 × CH_3), 7.37 (s, 1H, CH), 7.55–7.58 (m, 5H, Ar), 7.92 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.3 (t), 23.3 (t), 43.2 (2 × q), 100.7 (s), 121.2 (s), 123.2 (2 × d), 127.6 (d), 129.3 (2 × d), 138.1 (d), 138.6 (s), 146.3 (s), 147.7 (d), 181.5 (s). Anal calcd for $C_{16}H_{17}N_3O$ (267.33): C, 71.89; H, 6.41; N, 15.72. Found: C, 71.78; H, 6.60; N, 15.54.

*5.1.2.4. 1-Benzyl-5-[(dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-one (**9d**).* This compound was obtained from **8d**. Brown oil; yield: 82% (Method A), 95% (Method B); IR cm^{-1}: 1668 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.73–2.93 (m, 4H, 2 × CH_2), 2.89 (s, 6H, 2 × CH_3), 5.36 (s, 2H, CH_2), 7.13–7.36 (m, 5H, Ar), 7.79 (s, 1H, CH), 7.84 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.9 (t), 27.3 (t), 40.7 (2 × q), 61.7 (t), 108.5 (s), 117.5 (s), 127.4 (2 × d), 128.3 (d), 128.7 (2 × d), 131.9 (s), 136.5 (d), 146.7 (d), 162.3 (s), 180.8 (s). Anal calcd for $C_{17}H_{19}N_3O$ (281.35): C, 72.57; H, 6.81; N, 14.94. Found: C, 72.33; H, 7.02; N, 14.99.

*5.1.2.5. 1-(2-Chlorophenyl)-5-[(dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-one (**9e**).* This compound was obtained from **8e**. Brown solid; yield: 84% (Method A), 98% (Method B); m.p. 147–148 °C; IR cm^{-1}: 1637 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.58 (t, J = 6.6 Hz, 2H, CH_2), 2.96 (t, J = 6.6 Hz, 2H, CH_2), 3.06 (s, 6H, 2 × CH_3), 7.38 (s, 1H, CH), 7.54–7.76 (m, 4H, Ar), 7.92 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 21.0 (t), 23.1 (t), 43.2 (2 × q), 100.6 (s), 120.1 (s), 128.3 (d), 129.6 (d), 130.3 (d), 130.4 (s), 131.2 (d), 135.7 (s), 138.1 (d), 148.0 (d), 148.3 (s), 181.3 (s). Anal calcd for $C_{16}H_{16}ClN_3O$ (301.77): C, 63.68; H, 5.34; N, 13.92. Found: C, 63.80; H, 5.43; N, 14.18.

*5.1.2.6. 1-(3-Chlorophenyl)-5-[(dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-one (**9f**).* This compound was obtained from **8f**. Brown oil; yield: 70% (Method A), 95% (Method B); IR cm^{-1}: 1639 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.91–3.03 (m, 4H, 2 × CH_2), 3.08 (s, 6H, 2 × CH_3), 7.37 (s, 1H, CH), 7.53–7.69 (m, 4H, Ar), 7.95 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.2 (t), 23.3 (t), 43.2 (2 × q), 100.5 (s), 121.5 (s), 121.7 (d), 122.8 (d), 127.5 (d), 131.1 (d), 133.6 (s), 138.6 (d), 139.8 (s), 146.7 (s), 147.8 (d), 181.4 (s). Anal calcd for $C_{16}H_{16}ClN_3O$ (301.77): C, 63.68; H, 5.34; N, 13.92. Found: C, 63.77; H, 5.21; N, 13.96.

*5.1.2.7. 1-(4-Chlorophenyl)-5-[(dimethylamino)methylidene]-1,5,6,7-tetrahydro-4H-indazol-4-one (**9g**).* This compound was obtained from **8g**. Brown oil; yield: 70% (Method A), 95% (Method B); IR cm^{-1}: 1648 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.89–3.01 (m, 4H, 2 × CH_2), 3.08 (s, 6H, 2 × CH_3), 7.37 (s, 1H, CH), 7.58 (d, J = 8.5 Hz, 2H, H-3″and H-5″), 7.62 (d, J = 8.5 Hz, 2H, H-2″ and H-6″), 7.94 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.2 (t), 23.3 (t), 43.2 (2 × q), 100.5 (s), 121.8 (s), 124.7 (2 × d), 129.3 (2 × d), 132.6 (s), 138.4 (d), 147.8 (d), 149.4 (s), 156.6 (s), 181.4 (s). Anal calcd for $C_{16}H_{16}ClN_3O$ (301.77): C, 63.68; H, 5.34; N, 13.92. Found: C, 63.55; H, 5.23; N, 14.11.

*5.1.2.8. 5-[(Dimethylamino)methylidene]-1-(4-metoxyphenyl)-1,5,6,7-tetrahydro-4H-indazol-4-one (**9h**).* This compound was obtained from **8h**. Brown solid; yield: 71% (Method A), 96% (Method B); m.p. 151–152 °C; IR cm^{-1}: 1666 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.87–2.95 (m, 4H, 2 × CH_2), 3.07 (s, 6H, 2 × CH_3), 3.82 (s, 3H, CH_3), 7.09 (d, J = 8.7 Hz, 2H, H-3″ and H-5″), 7.36 (s, 1H, CH), 7.50 (d, J = 8.7 Hz, 2H, H-2″ and H-6″), 7.88 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.0 (t), 23.3 (t), 43.2 (2 × q), 55.4 (q), 100.8 (s), 114.4 (2 × d), 120.7 (s), 124.8 (2 × d), 131.7 (d), 137.7 (s), 146.1 (d), 147.6 (s), 158.5 (s), 181.5 (s). Anal calcd for $C_{17}H_{19}N_3O_2$ (297.35): C, 68.67; H, 6.44; N, 14.13. Found: C, 68.91; H, 6.19; N, 14.32.

*5.1.3. General procedure for the preparation of 3,7-substituted 1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-ones (**10a-j**)*

To a solution of the suitable enaminoketons **9a-h** (20 mmol) in anhydrous ethanol (30 mL), a solution of the proper cyanomethylene compound (20 mmol) in anhydrous ethanol (30 mL) was added dropwise under nitrogen atmosphere. Then the reaction mixture was heated at reflux for 24 h. Upon cooling, a solid separated which was filtered, dried (Na_2SO_4) and recrystallized from ethanol.

*5.1.3.1. 3-(Phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**10a**).* This compound was obtained from reaction of **9a** with phenylsulfonylacetonitrile. White solid; yield: 60%; m.p. >410 °C; IR cm^{-1}: 3110 (NH), 3078 (NH), 1653 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.75–2.91 (m, 4H, 2 × CH_2), 7.55–7.67 (m, 3H, H-3′, H-4′ and H-5′), 7.96 (dd, J = 7.1, 1.5 Hz, 2H, H-2′ and H-6′), 8.06 (s, 1H, H-9), 8.20 (s, 1H, H-4), 12.60 (s, 1H, NH), 13.17 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.7 (t), 25.5 (t), 110.1 (s), 121.9 (s), 127.8 (2 × d), 128.7 (2 × d), 129.3 (d), 133.0 (d), 140.7 (s), 143.9 (d), 144.7 (s), 145.2 (s), 151.6 (s), 157.2 (s). Anal. $C_{16}H_{13}N_3O_3S$ (327.36): C, 58.70; H, 4.00, N, 12.84. Found: C, 58.85; H, 4.33, N, 12.68.

*5.1.3.2. 7-Methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo [3,4-h]quinolin-2-one (**10b**).* This compound was obtained from reaction of **9b** with phenylsulfonylacetonitrile. Yellow solid; yield: 80%; m.p. 380–382 °C; IR cm^{-1}: 3157 (NH), 1631 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.49–2.51 (m, 2H, CH_2), 2.90 (s, 3H, CH_3), 2.66–2.78 (m, 2H, CH_2), 7.53–7.69 (m, 3H, H-3′, H-4′ and H-5′), 7.06 (dd, J = 7.5, 1.4 Hz, 2H, H-2′ and H-6′), 8.05 (1H, s, H-9), 8.17 (1H, s, H-4), 12.56 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.0 (t), 24.9 (t), 35.9 (q), 107.3 (s), 110.7 (s), 121.1 (s), 127.8 (2 × d),

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128.7 (2 × d), 133.0 (d), 135.3 (d), 140.9 (s), 143.6 (d), 143.8 (s), 144.6 (s), 157.2 (s). Anal. $C_{17}H_{15}N_3O_3S$ (341.38): C, 59.81; H, 4.43 N, 12.31. Found: C, 60.05; H, 4.25; N, 12.40.

*5.1.3.3. 7-Phenyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo [3,4-h]quinolin-2-one (**10c**).* This compound was obtained from reaction of **9c** with phenylsulfonylacetonitrile. Brown solid; yield: 63%; m.p. 392–393 °C; IR cm^{-1}: 3105 (NH), 1643 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.92 (t, J = 7.2 Hz, 2H, CH_2), 3.06 (t, J = 7.2 Hz, 2H, CH_2), 7.43–7.71 (m, 8H, Ar), 7.99 (dd, J = 6.6, 1.5 Hz, 2H, H-2′ and H-6′), 8.24 (s, 1H, H-9), 8.35 (s, 1H, H-4), 12.73 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.6 (t), 25.2 (t), 108.0 (s), 112.5 (s), 122.1 (s), 123.4 (2 × d), 127.8 (2 × d), 128.1 (d), 128.7 (2 × d), 129.4 (2 × d), 133.1 (d), 137.1 (d), 138.2 (s), 140.7 (s), 143.8 (d), 144.2 (s), 144.6 (s), 157.2 (s). Anal. $C_{22}H_{17}N_3O_3S$ (403.45): C, 65.49; H, 4.25; N, 10.42. Found: C, 65.55; H, 4.09; N, 10.31.

*5.1.3.4. 7-Benzyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo [3,4-h]quinolin-2-one (**10d**).* This compound was obtained from reaction of **9d** with phenylsulfonylacetonitrile. White solid; yield: 88%; m.p. 285–287 °C; IR cm^{-1}: 3089 (NH), 1631 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.89–2.94 (m, 4H, 2 × CH_2), 5.37 (s, 2H, CH_2), 7.16 (dd, J = 7.5, 2.0 Hz, 2H, Ar), 7.28–7.35 (m, 3H, Ar), 7.53–7.66 (m, 3H, Ar), 7.96 (dd, J = 8.0, 1.7 Hz, 2H, H-2′ and H-6′), 8.14 (s, 1H, H-9), 8.18 (s, 1H, H-4), 12.62 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.3 (t), 25.0 (t), 52.3 (t), 107.3 (s), 111.1 (s), 121.4 (s), 127.4 (2 × d), 127.6 (d), 127.7 (2 × d), 128.5 (2 × d), 128.6 (2 × d), 133.0 (d), 135.9 (s), 136.4 (s), 140.9 (d), 143.7 (d), 144.6 (s), 148.2 (s), 157.2 (s). Anal. $C_{23}H_{19}N_3O_3S$ (417.48): C, 66.17; H, 4.59; N, 10.07. Found: C, 66.34; H, 4.77; N, 10.21.

*5.1.3.5. 7-(2-Chlorophenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**10e**).* This compound was obtained from reaction of **9e** with phenylsulfonylacetonitrile. White solid; yield: 65%; m.p. 349–350 °C; IR cm^{-1}: 3238 (NH), 1637 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.73 (t, J = 7.6 Hz, 2H, CH_2), 2.92 (t, J = 7.6 Hz, 2H, CH_2), 7.52–7.78 (m, 7H, Ar), 7.98 (dd, J = 8.0, 1.7 Hz, 2H, H-2′ and H-6′), 8.23 (s, 1H, H-9), 8.35 (s, 1H, H-4), 12.80 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.8 (t), 24.9 (t), 111.4 (s), 122.2 (s), 127.8 (2 × d), 128.5 (d), 128.9 (2 × d), 129.5 (d), 130.4 (d), 130.5 (s), 131.7 (d), 133.1 (d), 135.3 (s), 137.1 (d), 138.2 (s), 140.8 (s), 143.9 (d), 144.3 (s), 146.2 (s), 157.2 (s). Anal. $C_{22}H_{16}ClN_3O_3S$ (437.90): C, 60.34; H, 3.68, N, 9.60. Found: C, 60.48; H, 3.39, N, 9.78.

*5.1.3.6. 7-(3-Chlorophenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**10f**).* This compound was obtained from reaction of **9f** with phenylsulfonylacetonitrile. Gray solid; yield: 68%; m.p. 379–381 °C; IR cm^{-1}: 3103 (NH), 1649 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.93 (t, J = 6.8 Hz, 2H, CH_2), 3.09 (t, J = 6.8 Hz, 2H, CH_2), 7.59–7.71 (m, 7H, Ar), 7.98 (d, J = 6.8 Hz, 2H, H-2′ and H-6′), 8.25 (s, 1H, H-9), 8.37 (s, 1H, H-4), 12.76 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 22.5 (t), 25.1 (t), 122.1 (d), 123.2 (d), 127.9 (2 × d), 128.1 (d), 128.7 (2 × d), 131.1 (d), 133.1 (d), 133.7 (s), 135.5 (s), 136.0 (s), 137.5 (d), 139.3 (s), 140.7 (s), 142.9 (s), 143.8 (d), 144.6 (s), 151.4 (s), 157.2 (s). Anal. $C_{22}H_{16}ClN_3O_3S$ (437.90): C, 60.34; H, 3.68; N, 9.60. Found: C, 60.18; H, 3.77; N, 9.79.

*5.1.3.7. 7-(4-Chlorophenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**10g**).* This compound was obtained from reaction of **9g** with phenylsulfonylacetonitrile. Yellow solid; yield: 65%; m.p. >410 °C; IR cm^{-1}: 3086 (NH), 1649 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.92–3.07 (m, 4H, 2 × CH_2), 7.10 (d, J = 7.4 Hz, 2H, H-3′ and H-5′), 7.55–7.68 (m, 5H, Ar), 7.98 (dd, J = 7.4, 1.7 Hz, 2H, H-2′ and H-6′), 8.25 (s, 1H, H-9), 8.35 (s, 1H, H-4), 12.75 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.5 (t), 25.1 (t), 122.3 (s), 125.1 (2 × d), 125.9 (s), 127.9 (2 × d), 128.7 (2 × d), 129.4 (2 × d), 131.7 (s), 132.5 (s), 133.1 (d), 137.0 (s), 137.4 (d), 140.7 (s), 157.6 (d), 144.4 (s), 144.5 (s), 157.3 (s). Anal. $C_{22}H_{16}ClN_3O_3S$ (437.90): C, 60.34; H, 3.68; N, 9.60. Found: C, 60.52; H, 3.55; N, 9.40.

*5.1.3.8. 7-(4-Methoxyphenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**10h**).* This compound was obtained from reaction of **9h** with phenylsulfonylacetonitrile. Yellow solid; yield: 73%; m.p. 387–389 °C; IR cm^{-1}: 3078 (NH), 1645 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.90–2.99 (m, 4H, 2 × CH_2), 3.82 (s, 3H, CH_3), 7.10 (d, J = 8.7 Hz, 2H, H-3″ and H-5″), 7.50 (d, J = 8.7 Hz, 2H, H-2″ and H-6″), 7.58–7.67 (m, 3H, Ar), 7.98 (d, J = 7.4, 1.6 Hz, 2H, H-2′ and H-6′), 8.19 (s, 1H, H-9), 8.28 (s, 1H, H-4), 12.59 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.4 (t), 25.3 (t), 55.5 (q), 113.5 (s), 114.4 (2 × d), 124.8 (s), 125.0 (2 × d), 125.2 (d), 127.8 (2 × d), 128.7 (2 × d), 131.3 (s), 132.9 (s), 136.7 (d), 140.9 (s), 143.2 (s), 143.9 (d), 156.7 (s), 157.8 (s), 158.8 (s). Anal. $C_{23}H_{19}N_3O_4S$ (433.48): C, 63.73; H, 4.42; N, 9.69. Found: C, 63.92; H, 4.38; N, 9.81.

*5.1.3.9. Ethyl 7-(4-chlorophenyl)-2-oxo-2,5,6,7-tetrahydro-1H-pyrazolo[3,4-h]quinolin-3-carboxylate (**10i**).* This compound was obtained from reaction of **9g** with ethyl cyanoacetate. Yellow solid; yield: 72%; m.p. 271–272 °C; IR cm^{-1}: 3409 (NH), 1690 (CO), 1645 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 1.27 (t, J = 7.1 Hz, 3H, CH_3), 2.86 (t, J = 7.0 Hz, 2H, CH_2), 3.05 (t, J = 7.0 Hz, 2H, CH_2), 4.21 (q, J = 7.1 Hz, 2H, CH_2), 7.55–7.73 (m, 4H, Ar), 8.03 (s, 1H, H-9), 8.35 (s, 1H, H-4), 12.38 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 14.3 (q), 20.9 (t), 25.6 (t), 59.7 (t), 109.1 (d), 125.1 (2 × d), 128.1 (s), 129.4 (2 × d), 132.6 (s), 135.4 (s), 136.5 (s), 137.2 (d), 144.1 (s), 147.4 (s), 152.1 (s), 156.3 (s), 162.6 (s). Anal calcd for $C_{19}H_{16}ClN_3O_3$ (369.80): C, 61.71; H, 4.36; N, 11.36. Found: C, 61.86; H, 4.16; N, 11.55.

*5.1.3.10. Ethyl 7-(4-methoxyphenyl)-2-oxo-2,5,6,7-tetrahydro-1H-pyrazolo[3,4-h]quinolin-3-carboxylate (**10j**).* This compound was obtained from reaction of **9h** with ethyl cyanoacetate. Yellow solid; yield: 80%; m.p. 275–276 °C; IR cm^{-1}: 3414 (NH), 1685 (CO), 1644 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 1.27 (t, J = 7.1 Hz, 3H, CH_3), 2.85 (t, J = 6.7 Hz, 2H, CH_2), 2.98 (t, J = 6.7 Hz, 2H, CH_2), 3.83 (s, 3H, CH_3), 4.21 (q, J = 7.1 Hz, 2H, CH_2), 7.10 (d, J = 8.9 Hz, 2H, H-3″ and H-5″), 7.51 (d, J = 8.9 Hz, 2H, H-2″ and H-6”), 8.03 (s, 1H, H-9), 8.31 (s, 1H, H-4), 12.11 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 14.3 (q), 20.5 (t), 21.4 (t), 55.5 (q), 59.8 (t), 105.9 (s), 114.4 (2 × d), 125.0 (2 × d), 131.3 (s), 133.1 (s), 136.6 (d), 139.0 (s), 142.4 (s), 143.6 (d), 155.3 (s), 158.7 (s), 159.9 (s), 165.2 (s). Anal calcd for $C_{20}H_{19}N_3O_4$ (365.38): C, 65.74; H, 5.24; N, 11.50. Found: C, 65.93; H, 5.11; N, 11.65.

*5.1.4. General procedure for the preparation of 3,7-disubstituted 1-methyl-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-ones (**11b-l**) and 2-methoxy-7-methyl-6,7-dihydro-5H-pyrazolo[3,4-h] quinolines (**12b-l**)*

To a solution of the suitable pyrazoloquinolinone **10b-l** (2 mmol) in anhydrous DMF (8 mL), a stoichiometric amount of NaH was added. The reaction mixture was stirred for 3 h at room temperature and then the appropriate halide (2 mmol) was added. After 24 h, it was poured onto crushed ice and the precipitate was filtered off, dried and purified by chromatography (DCM/AcOEt 95:5) to obtain N-methyl derivatives **11b-l** and O-methyl derivatives **12b-l**.

*5.1.4.1. 1,7-Dimethyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11b**).* This compound was obtained from reaction of **10b** with iodomethane. Yellow solid; yield: 58%; m.p. 264–266 °C; IR cm^{-1}: 1649 (CO); ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.91–2.98 (m, 2H, CH_2), 3.04–3.11 (m, 2H, CH_2),

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3.79 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 7.57–7.74 (m, 3H, H-3′, H-4′ and H-5′), 7.79 (s, 1H, H-9), 7.92 (dd, J = 7.3, 1.4 Hz, 2H, H-2′ and H-6′), 8.17 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 18.8 (t), 26.1 (t), 35.9 (q), 53.6 (q), 116.7 (s), 117.8 (s), 120.7 (s), 127.7 (2 × d), 129.0 (2 × d), 133.5 (d), 134.7 (d), 137.8 (d), 140.7 (s), 144.0 (s), 153.4 (s), 158.2 (s). Anal. $C_{18}H_{17}N_3O_3S$ (355.41): C, 60.83; H, 4.82; N, 11.82. Found: C, 60.98; H, 4.64; N, 12.18.

*5.1.4.2. 1-Methyl-7-phenyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11c**).* This compound was obtained from reaction of **10c** with iodomethane. Yellow solid; yield: 58%; m.p. 289–291 °C; IR cm^{-1}: 1649 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.89–3.01 (m, 4H, CH_2), 3.73 (s, 3H, CH_3), 7.48–7.70 (m, 8H, Ar), 7.98 (d, J = 7.1 Hz, 2H, H-2′ and H-6′), 8.26 (s, 1H, H-9), 8.16 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.5 (t), 26.9 (t), 33.4 (q), 109.8 (s), 113.1 (s), 121.1 (s), 123.7 (2 × d), 128.1 (2 × d), 128.3 (d), 128.7 (2 × d), 129.4 (2 × d), 133.1 (d), 137.8 (s), 139.4 (d), 140.5 (s), 141.5 (d), 145.2 (s), 147.2 (s), 156.5 (s). Anal. $C_{23}H_{19}N_3O_3S$ (417.48): C, 66.17; H, 4.59; N, 10.07. Found: C, 66.31; H, 4.48; N, 9.97.

*5.1.4.3. 7-Benzyl-1-methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11d**).* This compound was obtained from reaction of **10d** with iodomethane. Gray solid; yield: 68%; m.p. 204–206 °C; IR cm^{-1}: 1643 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.86–2.92 (m, 4H, 2 × CH_2), 3.67 (s, 3H, CH_3), 5.44 (s, 3H, CH_2), 7.19–7.35 (m, 5H, Ar), 7.53–7.69 (m, 3H, Ar), 7.98 (d, J = 7.0 Hz, 2H, H-2′ and H-6′), 8.12 (s, 1H, H-9), 8.21 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 19.3 (t), 26.8 (t), 33.2 (q), 52.3 (t), 109.2 (s), 111.7 (s), 120.3 (s), 127.4 (2 × d), 127.7 (d), 128.1 (2 × d), 128.6 (2 × d), 128.7 (2 × d), 133.0 (d), 136.3 (s), 138.3 (d), 140.5 (s), 141.5 (d), 145.7 (s), 147.6 (s), 156.4 (s). Anal. $C_{24}H_{21}N_3O_3S$ (431.51): C, 66.80; H, 4.91; N, 9.74. Found: C, 66.65; H, 5.04; N, 9.55.

*5.1.4.4. 7-(2-Chlorophenyl)-1-methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11e**).* This compound was obtained from reaction of **10e** with iodomethanecome. Yellow solid; yield: 58%; m.p. 213–214 °C; IR cm^{-1}: 1649 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.69 (t, J = 7.6 Hz, 2H, CH_2), 2.91 (t, J = 7.6 Hz, 2H, CH_2), 3.75 (s, 3H, CH_3), 7.54–7.80 (m, 7H, Ar), 7.98 (d, J = 6.6 Hz, 2H, H-2′ and H-6′), 8.25 (s, 1H, H-9), 8.37 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.9 (t), 26.7 (t), 33.4 (q), 109.5 (s), 112.1 (s), 121.1 (s), 128.1 (2 × d), 128.5 (d), 128.7 (2 × d), 129.6 (d), 130.3 (s), 130.4 (d), 131.7 (d), 133.1 (d), 135.1 (s), 139.3 (d), 140.5 (s), 141.7 (d), 147.2 (s), 147.3 (s), 156.4 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 61.34; H, 3.80; N, 9.42.

*5.1.4.5. 7-(3-Chlorophenyl)-1-methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11f**).* This compound was obtained from reaction of **10f** with iodomethane. Yellow solid; yield: 56%; m.p. 245–247 °C; IR cm^{-1}: 1651 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.90 (t, J = 7.3 Hz, 2H, CH_2), 3.04 (t, J = 7.3 Hz, 2H, CH_2), 3.73 (s, 3H, CH_3), 7.54–7.73 (m, 7H, Ar), 7.99 (d, J = 8.3, 2H, H-2′ and H-6′), 8.27 (s, 1H, H-9), 8.38 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.4 (t), 26.9 (t), 33.5 (q), 110.0 (s), 113.4 (s), 121.3 (s), 122.4 (d), 123.5 (d), 128.2 (2 × d), 128.3 (d), 128.7 (2 × d), 131.1 (d), 133.1 (d), 133.7 (s), 139.0 (s), 139.7 (d), 140.4 (s), 141.6 (d), 145.6 (s), 147.0 (s), 156.5 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 60.97; H, 4.33; N, 9.17.

*5.1.4.6. 7-(4-Chlorophenyl)-1-methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11g**).* This compound was obtained from reaction of **10g** with iodomethane. Pale yellow solid; yield: 53%; m.p. 221–222 °C; IR cm^{-1}: 1653 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.89–3.02 (m, 4H, CH_2), 3.73 (s, 3H, CH_3), 7.54–7.70 (m, 7H, Ar), 7.98 (dd, J = 8.0, 1.7 Hz, 2H, H-2′ and H-6′), 8.27 (s, 1H, H-9), 8.37 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 20.4 (t), 26.9 (t), 33.4 (q), 109.9 (s), 113.3 (s), 121.2 (s), 125.4 (2 × d), 128.2 (2 × d), 128.7 (2 × d), 129.5 (2 × d), 132.8 (s), 133.1 (d), 136.7 (s), 139.6 (d), 140.4 (s), 141.6 (d), 145.4 (s), 147.1 (s), 156.5 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 61.22; H, 3.96; N, 9.45.

*5.1.4.7. 7-(4-Methoxyphenyl)-1-methyl-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11h**).* This compound was obtained from reaction of **10h** with iodomethane. Light green solid; yield: 59%; m.p. 236–237 °C; IR cm^{-1}: 1653 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.84–2.94 (m, 4H, 2 × CH_2), 3.73 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 7.12 (d, J = 8.9 Hz, 2H, H-3″ and H-5″), 7.52 (d, J = 8.9 Hz, 2H, H-2″ and H-6″), 7.58–7.67 (m, 3H, Ar), 7.98 (dd, J = 7.4, 1.6 Hz, 2H, H-2′ and H-6′), 8.25 (s, 1H, H-9), 8.30 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.3 (t), 33.4 (t), 38.2 (q), 55.1 (q), 109.7 (s), 112.6 (s), 114.5 (2 × d), 120.8 (s), 125.3 (2 × d), 128.1 (2 × d), 128.7 (2 × d), 130.9 (s), 133.1 (d), 139.0 (d), 140.5 (s), 141.5 (d), 145.1 (s), 147.4 (s), 156.5 (s), 159.0 (s). Anal. $C_{24}H_{21}N_3O_4S$ (447.51): C, 64.41; H, 4.73; N, 9.39. Found: C, 64.23; H, 4.99; N, 9.10.

*5.1.4.8. Ethyl 7-(4-chlorophenyl)-1-methyl-2-oxo-2,5,6,7-tetrahydro-1H-pyrazolo[3,4-h]quinoline-3-carboxylate (**11i**).* This compound was obtained from reaction of **10i** with iodomethane. Yellow solid; yield: 70%; m.p. 228–229 °C; IR cm^{-1}: 1679 (CO), 1656 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 1.40 (t, J = 7.1 Hz, 3H, CH_3), 2.84 (t, J = 7.4 Hz, 2H, CH_2), 2.98 (t, J = 7.4 Hz, 2H, CH_2), 3.93 (s, 3H, CH_3), 4.37 (q, J = 7.1 Hz, 2H, CH_2), 7.44 (d, J = 8.9 Hz, 2H, H-3″ and H-5″), 7.53 (d, J = 8.9 Hz, 2H, H-2″ and H-6″), 8.06 (s, 1H, H-9), 8.10 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 14.4 (q), 21.3 (t), 27.9 (t), 34.2 (q), 61.0 (t), 109.8 (s), 114.2 (s), 114.3 (s), 125.1 (2 × d), 129.7 (2 × d), 134.5 (s), 136.6 (s), 138.7 (d), 144.1 (s), 144.3 (d), 145.1 (s), 159.9 (s), 165.9 (s). Anal calcd for $C_{20}H_{18}ClN_3O_3$ (383.83): C, 62.58; H, 4.73; N, 10.95. Found: C, 62.71; H, 4.55; N, 11.19.

*5.1.4.9. Ethyl 7-(4-methoxyphenyl)-1-methyl-2-oxo-2,5,6,7-tetrahydro-1H-pyrazolo[3,4-h]quinoline-3-carboxylate (**11j**).* This compound was obtained from reaction of **10j** with iodomethane. Dark yellow solid; yield: 60%; m.p. 217–218 °C; IR cm^{-1}: 1652 (CO), 1684 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 1.40 (t, J = 7.1 Hz, 3H, CH_3), 2.78–2.98 (m, 4H, 2 × CH_2), 3.88 (s, 3H, CH_3), 3.93 (s, 3H, CH_3), 4.38 (q, J = 7.1 Hz, 2H, CH_2), 7.03 (d, J = 9.0 Hz, 2H, H-3″ and H-5″), 7.40 (d, J = 9.0 Hz, 2H, H-2″ and H-6″), 8.03 (s, 1H, H-9), 8.10 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 14.4 (q), 22.1 (t), 27.9 (t), 34.1 (q), 55.7 (q), 60.9 (t), 109.8 (s), 113.5 (s), 113.8 (s), 114.6 (2 × d), 125.4 (2 × d), 131.2 (s), 138.1 (d), 144.1 (s), 144.3 (d), 145.5 (s), 159.7 (s), 160.0 (s), 166.0 (s). Anal calcd for $C_{21}H_{21}N_3O_4$ (379.41): C, 66.48; H, 5.58; N, 11.08. Found: C, 66.59; H, 5.67; N, 10.95.

*5.1.4.10. 1-Benzyl-7-(4-chlorophenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11k**).* This compound was obtained from reaction of **10g** with benzyl bromide. Light yellow solid; yield: 30%; m.p. 151–152 °C; IR cm^{-1}: 1653 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.99–3.11 (m, 4H, 2 × CH_2), 5.44 (s, 2H, CH_2), 7.23–7.56 (m, 12H, Ar), 7.87 (d, J = 7.2 Hz, 2H, Ar), 8.08 (s, 1H, H-9), 8.23 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 21.3 (t), 27.3 (t), 68.6 (t), 119.4 (s), 119.8 (s), 120.4 (s), 124.6 (2 × d), 128.0 (d), 128.3 (2 × d), 128.4 (2 × d), 128.5 (2 × d), 128.6 (2 × d), 129.5 (2 × d), 132.9 (d), 133.7 (s), 136.3 (s), 137.4 (s), 137.6 (d), 138.0 (d), 140.8 (s), 142.6 (s), 152.9 (s), 158.5 (s). Anal calcd for $C_{29}H_{22}ClN_3O_3S$ (528.02): C, 65.97; H, 4.20; N, 7.96. Found: C, 65.81; H, 4.02; N, 8.17.

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*5.1.4.11. 1-Benzyl-7-(4-methoxyphenyl)-3-(phenylsulfonyl)-1,5,6,7-tetrahydro-2H-pyrazolo[3,4-h]quinolin-2-one (**11l**).* This compound was obtained from reaction of **10h** with benzyl bromide. Brown solid; yield: 28%; m.p. 134–135 °C; IR cm^{-1}: 1657 (CO); ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.89–3.05 (m, 4H, 2 × CH_2), 3.86 (s, 3H, CH_3), 5.53 (s, 2H, CH_2), 6.98–7.04 (m, 4H, Ar), 7.26–7.33 (m, 4H, Ar), 7.46–7.75 (m, 5H, Ar and H-9), 8.15 (d, J = 6.7 Hz, 2H, H-2′ and H-6′), 8.38 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 21.0 (t), 28.3 (t), 55.7 (q), 71.8 (t), 109.9 (s), 112.5 (s), 114.6 (2 × d), 123.5 (s), 125.3 (2 × d), 127.5 (2 × d), 128.6 (2 × d), 128.9 (2 × d), 129.2 (2 × d), 130.9 (d), 131.0 (s), 133.1 (d), 135.0 (s), 138.3 (d), 140.3 (s), 142.5 (d), 144.5 (s), 147.5 (s), 157.5 (s), 159.7 (s). Anal calcd for $C_{30}H_{25}N_3O_4S$ (523.60): C, 68.82; H, 4.81; N, 8.03. Found: C, 68.76; H, 4.99; N, 8.16.

*5.1.4.12. 2-Methoxy-7-methyl-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12b**).* This compound was obtained from reaction of **10b** with iodomethane. White solid; yield: 35%; m.p. 199–201 °C; ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.80–2.92 (m, 4H, 2 × CH_2), 3.66 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 7.52–7.69 (m, 3H, H-3′, H-4′ and H-5′), 7.96 (dd, J = 7.3, 1.4 Hz, 2H, H-2′ and H-6′), 8.04 (s, 1H, H-9), 8.21 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.0 (t), 26.7 (t), 33.2 (q), 36.2 (q), 109.1 (s), 111.3 (s), 120.1 (s), 128.1 (2 × d), 128.6 (2 × d), 133.1 (d), 137.7 (d), 140.6 (s), 141.5 (d), 145.8 (s), 147.8 (s), 156.4 (s). Anal. $C_{18}H_{17}N_3O_3S$ (355.41): C, 60.83; H, 4.82; N, 11.82. Found: C, 60.95; H, 4.60; N, 12.10.

*5.1.4.13. 2-Methoxy-7-phenyl-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12c**).* This compound was obtained from reaction of **10c** with iodomethanecome. White solid; yield: 30%; m.p. 197–199 °C; ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.98–3.11 (m, 4H, 2 × CH_2), 3.90 (s, 3H, CH_3), 7.46–7.72 (m, 8H, Ar), 7.96 (dd, J = 7.4, 1.6 Hz, 2H, H-2′ and H-6′), 8.11 (s, 1H, H-9), 8.25 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.5 (t), 26.3 (t), 53.7 (q), 118.5 (s), 118.6 (s), 121.3 (s), 123.1 (2 × d), 127.8 (2 × d), 129.1 (2 × d), 129.3 (d), 129.4 (2 × d), 133.5 (d), 136.8 (d), 137.9 (d), 138.5 (s), 140.5 (s), 145.7 (s), 147.6 (s), 156.4 (s). Anal. $C_{23}H_{19}N_3O_3S$ (417.48): C, 66.17; H, 4.59; N, 10.07. Found: C, 66.02; H, 4.68; N, 10.23.

*5.1.4.14. 7-Benzyl-2-methoxy-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12d**).* This compound was obtained from reaction of **10d** with iodomethane. White solid; yield: 30%; m.p. 123–124 °C; ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.90–3.10 (m, 4H, 2 × CH_2), 3.86 (s, 3H, CH_3), 5.38 (s, 3H, CH_2), 7.17–7.34 (m, 5H, Ar), 7.57–7.70 (m, 3H, Ar), 7.87–7.93 (m, 3H, H-2′ and H-6′, H-9), 8.16 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 19.1 (t), 26.1 (t), 52.2 (t), 53.6 (q), 117.2 (s), 118.0 (s), 120.8 (s), 127.2 (2 × d), 127.6 (d), 127.8 (2 × d), 128.6 (2 × d), 129.0 (2 × d), 133.5 (d), 135.4 (d), 136.8 (s), 137.9 (d), 140.7 (s), 144.1 (s), 153.1 (s), 158.3 (s). Anal. $C_{24}H_{21}N_3O_3S$ (431.51): C, 66.80; H, 4.91; N, 9.74. Found: C, 66.99; H, 4.75; N, 9.88.

*5.1.4.15. 7-(2-Chlorophenyl)-2-methoxy-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12e**).* This compound was obtained from reaction of **10e** with iodomethane. White solid; yield: 33%; m.p. 187–188 °C; ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 2.77 (t, J = 7.8 Hz, 2H, CH_2), 3.10 (t, J = 7.8 Hz, 2H, CH_2), 3.91 (s, 3H, CH_3), 7.56–7.77 (m, 7H, Ar), 7.92–7.97 (m, 2H, H-2′ and H-6′), 8.11 (s, 1H, H-9), 8.23 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 19.6 (t), 26.1 (t), 53.8 (q), 117.6 (s), 118.6 (s), 121.1 (s), 122.2 (s), 127.8 (2 × d), 128.4 (d), 129.1 (2 × d), 129.5 (d), 130.4 (d), 131.4 (d), 133.5 (d), 135.6 (s), 136.7 (d), 138.1 (d), 140.6 (s), 145.6 (s), 152.8 (s), 158.4 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 61.25; H, 3.83; N, 9.05.

*5.1.4.16. 7-(3-Chlorophenyl)-2-methoxy-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12f**).* This compound was obtained from reaction of **10f** with iodomethane. Yellow solid; yield: 35%; m.p. 220–222 °C; ^1H NMR (DMSO-d_6, 200 MHz, ppm): δ 3.12–3.15 (m, 4H, 2 × CH_2), 3.90 (s, 3H, CH_3), 7.54–7.72 (m, 7H, Ar), 7.93–7.97 (m, 2H, H-2′ and H-6′), 8.14 (s, 1H, H-9), 8.25 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.4 (t), 26.2 (t), 53.8 (q), 118.8 (s), 119.0 (s), 121.4 (s), 121.8 (d), 122.9 (d), 127.6 (d), 127.8 (2 × d), 129.1 (2 × d), 131.1 (d), 133.6 (d), 133.7 (s), 137.3 (d), 138.0 (s), 139.7 (d), 140.6 (s), 144.0 (s), 152.6 (s), 158.3 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 61.01; H, 3.84; N, 9.55.

*5.1.4.17. 7-(4-Chlorophenyl)-2-methoxy-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12g**).* This compound was obtained from reaction of **10g** with iodomethane. White solid; yield: 30%; m.p. 257–259 °C; ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.98–3.09 (m, 4H, 2 × CH_2), 3.90 (s, 3H, CH_3), 7.58–7.65 (m, 7H, Ar), 7.94 (dd, J = 7.6, 1.7 Hz, 2H, H-2′ and H-6′), 8.13 (s, 1H, H-9), 8.25 (s, 1H, H-4); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 20.4 (t), 26.3 (t), 53.8 (q), 118.7 (s), 118.8 (s), 121.3 (s), 124.8 (2 × d), 127.8 (2 × d), 129.1 (2 × d), 129.4 (2 × d), 133.1 (s), 133.6 (d), 137.1 (d), 137.4 (s), 138.0 (d), 140.6 (s), 143.8 (s), 152.6 (s), 158.3 (s). Anal. $C_{23}H_{18}ClN_3O_3S$ (451.93): C, 61.13; H, 4.01; N, 9.30. Found: C, 60.88; H, 4.27; N, 9.13.

*5.1.4.18. 2-Methoxy-7-(4-methoxyphenyl)-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12h**).* This compound was obtained from reaction of **10h** with iodomethane. White solid; yield: 36%; m.p. 186–187 °C; ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.98–3.03 (m, 4H, 2 × CH_2), 3.87 (s, 3H, CH_3), 3.98 (s, 3H, CH_3), 7.00 (d, J = 8.8 Hz, 2H, H-3″ and H-5″), 7.40 (d, J = 8.8 Hz, 2H, H-2″ and H-6″), 7.50–7.55 (m, 3H, H-3′, H-4′ and H-5′), 7.98 (dd, J = 7.6, 1.7 Hz, 2H, H-2′ and H-6′), 8.06 (s, 1H, H-9), 8.19 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 21.1 (t), 27.4 (t), 54.0 (q), 55.6 (q), 114.5 (2 × d), 119.0 (s), 119.1 (s), 120.3 (s), 125.0 (2 × d), 128.4 (2 × d), 128.6 (2 × d), 132.0 (s), 133.1 (d), 136.9 (d), 137.9 (d), 141.0 (s), 142.6 (s), 153.4 (s), 130.3 (s), 159.2 (s). Anal. $C_{24}H_{21}N_3O_4S$ (447.51): C, 64.41; H, 4.73; N, 9.39. Found: C, 64.54; H, 4.60; N, 9.47.

*5.1.4.19. Ethyl 7-(4-chlorophenyl)-2-methoxy-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline-3-carboxylate (**12i**).* This compound was obtained from reaction of **10i** with iodomethane. White solid; yield: 22%; m.p. 156–157 °C; IR cm^{-1}: 1710 (CO); ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 1.39 (t, J = 7.1 Hz, 3H, CH_3), 2.95–3.10 (m, 4H, 2 × CH_2), 4.12 (s, 3H, CH_3), 4.36 (q, J = 7.1 Hz, 2H, CH_2), 7.42–7.50 (m, 4H, Ar), 8.02 (s, 1H, H-9), 8.15 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 14.4 (q), 21.5 (t), 27.3 (t), 54.1 (q), 60.9 (t), 109.8 (s), 119.7 (s), 120.3 (s), 124.6 (2 × d), 129.5 (2 × d), 133.5 (s), 137.5 (d), 137.6 (s), 140.2 (d), 142.3 (s), 151.3 (s), 162.0 (s), 165.1 (s). Anal calcd for $C_{20}H_{18}ClN_3O_3$ (383.83): C, 62.58; H, 4.73; N, 10.95. Found: C, 62.37; H, 4.65; N, 11.07.

*5.1.4.20. Ethyl 2-methoxy-7-(4-methoxyphenyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline-3-carboxylate (**12j**).* This compound was obtained from reaction of **10j** with iodomethane. Pale brown solid; yield: 35%; m.p. 154–155 °C; IR cm^{-1}: 1711 (CO); ^1H NMR ($CDCl_3$, 200 MHz, ppm): δ 1.39 (t, J = 7.1 Hz, 3H, CH_3), 2.91–3.09 (m, 4H, 2 × CH_2), 3.87 (s, 3H, CH_3), 4.12 (s, 3H, CH_3), 4.36 (q, J = 7.1 Hz, 2H, CH_2), 7.01 (d, J = 8.9 Hz, 2H, H-3″ and H-5″), 7.41 (d, J = 8.9 Hz, 2H, H-2″ and H-6″), 8.01 (s, 1H, H-9), 8.12 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 14.4 (q), 22.3 (t), 27.3 (t), 54.1 (q), 55.6 (q), 60.8 (t), 109.4 (s), 114.4 (2 × d), 119.6 (s), 119.8 (s), 125.0 (2 × d), 130.9 (s), 132.2 (s), 136.8 (d), 140.2 (d), 142.4 (s), 159.1 (s), 162.0 (s), 165.1 (s). Anal calcd for $C_{21}H_{21}N_3O_4$ (379.41): C, 66.48; H, 5.58; N, 11.08. Found: C, 66.37; H, 5.72; N, 10.88.

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*5.1.4.21. 2-(Benzyloxy)-7-(4-chlorophenyl)-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12k**).* This compound was obtained from reaction of **10g** with benzyl bromide. White solid; yield: 64%; m.p. 216–217 °C; ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 2.99–3.11 (m, 4H, 2 × CH_2), 5.44 (s, 2H, CH_2), 7.23–7.56 (m, 12H, Ar), 7.87 (d, J = 7.2 Hz, 2H, H-2′ and H-6′), 8.08 (s, 1H, H-9), 8.23 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 21.3 (t), 27.3 (t), 68.6 (t), 119.4 (s), 119.8 (s), 120.4 (s), 124.6 (2 × d), 128.0 (d), 128.3 (2 × d), 128.4 (2 × d), 128.5 (2 × d), 128.6 (2 × d), 129.5 (2 × d), 132.9 (d), 133.7 (s), 136.3 (s), 137.4 (s), 137.6 (d), 138.0 (d), 140.8 (s), 142.6 (s), 152.9 (s), 158.5 (s). Anal calcd for $C_{29}H_{22}ClN_3O_3S$ (528.02): C, 65.97; H, 4.20; N, 7.96. Found: C, 66.08; H, 4.02; N, 7.85.

*5.1.4.22. 2-(Benzyloxy)-7-(4-methoxyphenyl)-3-(phenylsulfonyl)-6,7-dihydro-5H-pyrazolo[3,4-h]quinoline (**12l**).* This compound was obtained from reaction of **10h** with benzyl bromide. Pale green solid; yield: 55%; m.p. 188–189 °C; ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 3.01–3.05 (m, 4H, 2 × CH_2), 3.87 (s, 3H, CH_3), 5.44 (s, 2H, CH_2), 7.01 (d, J = 9.0 Hz, 2H, H-3″ and H-5″), 7.24–7.55 (m, 10H, Ar), 7.87 (d, J = 7.2 Hz, 2H, H-2′ and H-6′), 8.06 (s, 1H, H-9), 8.21 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 22.1 (t), 27.4 (t), 55.6 (q), 68.5 (t), 114.5 (2 × d), 119.0 (s), 119.1 (s), 120.5 (s), 125.0 (2 × d), 127.9 (d), 128.3 (2 × d), 128.4 (2 × d), 128.5 (2 × d), 128.6 (2 × d), 132.1 (s), 132.9 (d), 136.4 (s), 137.0 (d), 138.0 (d), 140.9 (s), 142.6 (s), 153.3 (s), 158.4 (s), 159.2 (s). Anal calcd for $C_{30}H_{25}N_3O_4S$ (523.60): C, 68.82; H, 4.81; N, 8.03. Found: C, 68.94; H, 4.69; N, 8.32.

*5.1.5. General procedure for the preparation of 1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-ones (**13a-f**)*

To a solution of the proper 1,5,6,7-tetrahydro-2*H*-pyrazolo[3,4-*h*]quinoline **10g,h**, **11g,h** and **12g,h** (30 mmol) in anhydrous dioxane (100 mL), DDQ was added (27.24 g, 120 mmol). The reaction mixture was heated under reflux for 24 h. Then the solvent was removed under reduced pressure and the crude was purified by chromatography with a SEPACORE BÜCHI (DCM/AcOEt 95:5).

*5.1.5.1. 7-(4-Chlorophenyl)-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13a**).* This compound was obtained from reaction of **10g**. Brown solid; yield: 50%; m.p. 309–310 °C; IR cm^{-1}: 3240 (NH), 1653 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 7.63–7.84 (m, 8H, Ar), 8.02–8.06 (m, 3H, Ar), 8.97 (s, 1H, H-9), 9.09 (s, 1H, H-4), 13.09 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 101.4 (s), 106.8 (d), 113.8 (s), 124.8 (2 × d), 128.2 (2 × d), 128.9 (2 × d), 129.2 (s), 129.7 (2 × d), 132.0 (s), 133.6 (d), 135.7 (d), 137.6 (s), 139.8 (s), 140.6 (s), 145.1 (d), 150.8 (s), 151.8 (d), 157.1 (s). Anal calcd for $C_{22}H_{14}ClN_3O_3S$ (435.88): C, 60.62; H, 3.24; N, 9.64. Found: C, 60.87; H, 3.11; N, 9.88.

*5.1.5.2. 7-(4-Methoxyphenyl)-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13b**).* This compound was obtained from reaction of **10h**. Dark yellow solid; yield: 60%; m.p. 373–374 °C; IR cm^{-1}: 3248 (NH), 1649 (CO); ^{1}H NMR (DMSO-d_6, 200 MHz, ppm): δ 3.86 (s, 3H, CH_3), 7.17 (d, J = 9.0 Hz, 2H, H-3″ and H-5″), 7.50 (d, J = 9.0 Hz, 2H, H-2″ and H-6″), 7.58–7.72 (m, 4H, Ar), 7.95–8.06 (m, 3H, H-3′, H-4′ and H-5′), 8.91 (s, 1H, H-9), 9.07 (s, 1H, H-4), 13.07 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz, ppm): δ 55.5 (q), 106.7 (d), 110.8 (s), 112.2 (s), 114.8 (2 × d), 125.0 (2 × d), 127.2 (s), 128.2 (2 × d), 128.9 (2 × d), 129.4 (d), 131.7 (s), 133.6 (d), 135.0 (d), 136.8 (s), 139.9 (s), 140.8 (s), 145.2 (d), 157.1 (s), 158.7 (s). Anal calcd for $C_{23}H_{17}N_3O_4S$ (431.46): C, 64.03; H, 3.97; N, 9.74. Found: C, 64.22; H, 4.08; N, 9.63.

*5.1.5.3. 7-(4-Chlorophenyl)-1-methyl-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13c**).* This compound was obtained from reaction of **11g**. White solid; yield: 93%; m.p. 264–265 °C; IR cm^{-1}: 1731 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 4.10 (s, 3H, CH_3), 7.50–7.73 (m, 9H, Ar), 8.19 (dd, J = 8.1, 1.5 Hz, 2H, H-2′ and H-6′), 8.63 (s, 1H, H-9), 8.90 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 33.7 (q), 106.8 (d), 112.3 (s), 114.0 (s), 125.3 (2 × d), 127.7 (s), 128.7 (2 × d), 129.2 (2 × d), 130.0 (2 × d), 130.4 (d), 133.5 (d), 134.2 (s), 136.5 (d), 137.2 (s), 139.3 (s), 139.6 (s), 142.1 (s), 143.5 (d), 157.3 (s). Anal calcd for $C_{23}H_{16}ClN_3O_3S$ (449.91): C, 61.40; H, 3.58; N, 9.34. Found: C, 61.27; H, 3.45; N, 9.49.

*5.1.5.4. 7-(4-Methoxyphenyl)-1-methyl-3-(phenylsulfonyl)-1,7-dihydro-2H-pyrazolo[3,4-h]quinolin-2-one (**13d**).* This compound was obtained from reaction of **11h**. Pale yellow solid; yield: 95%; m.p. 232–233 °C; IR cm^{-1}: 1657 (CO); ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 3.91 (s, 3H, CH_3), 4.10 (s, 3H, CH_3), 7.44 (d, J = 8.9 Hz, 2H, H-3″ and H-5″), 7.45–7.68 (m, 7H, Ar), 8.18 (dd, J = 8.1, 1.6 Hz, 2H, H-2′ and H-6′), 8.60 (s, 1H, H-9), 8.90 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 33.7 (q), 55.7 (q), 107.2 (d), 112.0 (s), 113.6 (s), 114.9 (2 × d), 125.8 (2 × d), 127.2 (s), 128.7 (2 × d), 129.1 (2 × d), 129.9 (d), 131.6 (s), 133.5 (d), 135.9 (d), 139.4 (s), 139.7 (s), 142.4 (s), 143.6 (d), 157.4 (s), 159.7 (s). Anal calcd for $C_{24}H_{19}N_3O_4S$ (445.49): C, 64.71; H, 4.30; N, 9.43. Found: C, 64.56; H, 4.24; N, 9.60.

*5.1.5.5. 7-(4-Chlorophenyl)- 2-methoxy-3-(phenylsulfonyl)-7H-pyrazolo[3,4-h]quinoline (**13e**).* This compound was obtained from reaction of **12g**. White solid; yield: 65%; m.p. 245–246 °C; ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 4.14 (s, 3H, CH_3), 7.48–7.84 (m, 9H, Ar), 8.07 (d, J = 6.8 Hz, 2H, H-2′ and H-6′), 8.71 (s, 1H, H-9), 9.00 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 55.3 (q), 110.2 (d), 119.1 (s), 121.0 (s), 122.9 (s), 124.6 (2 × d), 128.5 (d), 128.7 (2 × d), 128.8 (2 × d), 129.8 (2 × d), 133.4 (s), 133.5 (d), 136.1 (d), 138.0 (s), 140.4 (s), 140.6 (s), 141.0 (d), 145.0 (s), 158.4 (s). Anal calcd for $C_{23}H_{16}ClN_3O_3S$ (449.91): C, 61.40; H, 3.58; N, 9.34. Found: C, 61.58; H, 3.32; N, 9.61.

*5.1.5.6. 2-Methoxy-7-(4-methoxyphenyl)-3-(phenylsulfonyl)-7H-pyrazolo[3,4-h]quinoline (**13f**).* This compound was obtained from reaction of **12h**. Pale yellow solid; yield: 86%; m.p. 225–226 °C; ^{1}H NMR ($CDCl_3$, 200 MHz, ppm): δ 3.91 (s, 3H, CH_3), 4.14 (s, 3H, CH_3), 7.09 (d, J = 9.0 Hz, 2H, H-3″ and H-5″), 7.52–7.67 (m, 6H, Ar), 7.77 (d, J = 9.0 Hz, 1H, Ar), 8.04–8.09 (m, 2H, H-2′ and H-6′), 8.68 (s, 1H, H-9), 9.00 (s, 1H, H-4); ^{13}C NMR ($CDCl_3$, 50 MHz, ppm): δ 54.3 (q), 55.7 (q), 110.5 (d), 114.8 (2 × d), 118.9 (s), 121.4 (s), 122.4 (s), 125.2 (2 × d), 128.0 (d), 128.7 (2 × d), 128.8 (2 × d), 132.5 (s), 133.4 (d), 135.3 (d), 140.5 (s), 140.9 (s), 141.0 (d), 145.1 (s), 158.3 (s), 159.2 (s). Anal calcd for $C_{24}H_{19}N_3O_4S$ (445.49): C, 64.71; H, 4.30; N, 9.43. Found: C, 64.95; H, 4.08; N, 9.57.

5.2. Biology

5.2.1. Reagents

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

5.2.2. Irradiation procedure

HPW 125 Philips lamps (Wood's lamps), emitting mainly at λ = 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.

5.2.2.1. 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.

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5.2.3. Cell lines

Jurkat (human T-cell leukemia) and HL-60 (human promyelocytic leukemia) cells were cultured in RPMI-1640 medium; LoVo (human intestinal adenocarcinoma) cells were grown in Ham's F-12 medium, and MCF-7 (human breast adenocarcinoma) and A-549 (human lung carcinoma) cells were cultured in DMEM. All media were supplemented with penicillin G (115 U/mL), streptomycin (115 mg/mL), and 10% fetal bovine serum (Invitrogen, Milan, Italy).

5.2.3.1. Cellular antiproliferative tests and phototoxicity. Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 mL complete medium containing 5×10^3 cells. Plates were harvested at 37 °C in a humidified incubator (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 added into each well and incubated at 37 °C for 30 min and then irradiated (1.25, 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 [76]. Analogous phototoxicity experiments were carried out in the presence of different scavengers.

5.2.3.2. Colony formation assay. 10×10^3 A-549 cells were plated in dishes. After 24 h, cells were irradiated (2.5 J/cm^2) in the presence of the tested compounds at different concentrations. After irradiation, medium was replaced and cells were cultured for 1–2 weeks until well-defined colonies had formed (replacing culture medium every 2–3 days). Cells were stained with crystal violet as described in Ref. [6] and colonies of ≥50 cells were then counted visually. Data represented the average of the three values and error bars represented the SEM.

5.2.4. Generation of active oxygen species

Samples containing the test compounds (2.2×10^{-5} M), RNO (*N,N*-dimethyl-p-nitrosoaniline) (4×10^{-5} M) and imidazole (4×10^{-5} M) in phosphate buffer (pH 7.3) were irradiated with increasing UV-A doses and their absorbance at 440 nm was then measured for the determination of singlet oxygen. Data were expressed as percentage of RNO bleaching. Samples containing the tested compounds (10^{-5} M) and nitroblue tetrazolium (1.6×10^{-4} M) in carbonate buffer (pH 10) were irradiated with increasing UV-A doses. Their absorbance at 560 nm was measured to monitor superoxide anion formation [77].

5.2.5. Externalization of phosphatidylserine

Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured with a BD FACS Calibur 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 λ = 488 nm, and the emission filters were at λ = 525 and 585 nm [61].

5.2.6. Western blot analysis

Jurkat cells were irradiated (2.5 J/cm^2) in the presence of the tested compounds (1 μM), after 24 h, were centrifuged, and washed two times with ice cold phosphate-buffered saline (PBS). The pellet was resuspended in lysis buffer [35]. After the cells were lysed on ice for 20 min, lysates were centrifuged at 10,000 *g* at 4 °C for 20 min. The protein concentration in the supernatant was determined. Equal amounts of protein (20 μg) were resolved using 8–16% gradient polyacrylamide precast gels (Thermo Scientific) and transferred on a nitrocellulose Hybond-p membrane (GE Healthcare). Membranes were blocked with 5% skim milk powder in Tween-PBS for at least 2 h. Membranes were incubated with primary antibodies against caspase-3 and β-actin (all rabbit, 1:1000, Cell Signaling), for 2 h. Membranes were next incubated with peroxidase-labeled goat antirabbit IgG (1:3000, Cell Signaling) for 60 min. All membranes were visualized using ECL Advance (GE Healthcare) and exposed to Hyperfilm MP (GE Healthcare).

5.2.7. 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 [63]. Briefly, 24 h post-irradiation, Jurkat cells were collected by centrifugation and resuspended in HBSS containing JC-1 at 1 μM. Cells were then incubated at 37 °C for 10 min, and fluorescence was examined at 530 and 590 nm. The production of ROS was measured by flow cytometry using the fluorescence probes HE and DCFDA (both from Molecular Probes, USA); 24 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, and resuspended in HBSS. The fluorescence was recorded directly with the flow cytometer by using excitation at λ = 488 nm and emission at λ = 585 and 530 nm for HE and DCFDA, respectively [66].

5.2.8. Lysosomal stability assessment

Jurkat cells were assessed for lysosomal stability using the acridine orange (AO) uptake method [68]. After 24 h from the irradiation in the presence of test compounds, cells were stained with AO at 5 mg/mL at 37 °C for 15 min, washed, and then analyzed by flow cytometry using excitation at λ = 488 nm and detecting the emission at λ = 546 nm.

5.2.9. DNA frank strand breaks

pBR322 DNA samples (100 ng) dissolved in phosphate buffer 10 mM pH 7.2 were irradiated (UV-A dose: 7.5 J/cm^2) in the presence of the tested compound at [Compound]/[DNA] ratio = 3/1. After irradiation, two aliquots of sample were incubated at 37 °C with Fpg (formamydo pyrimidin glycosilase) and Endo III (Endonuclease III), respectively. The samples were loaded on 1% agarose gel and the run was carried out in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA) at 70 V for 4 h. The quantification of DNA bands was carried out as described previously [71].

5.2.10. Photoreaction of isolated proteins

Solutions of bovine serum albumin (BSA) (0.5 mg/mL) in phosphate buffer 10 mM were irradiated in the presence of the test compounds (10 μM) for various time in a quartz cuvette. Solutions of ribonuclease A (RNAseA), 0.5 mg/mL in phosphate buffer 10 mM were irradiated in the presence of the test compounds for various time in a quartz cuvette. At each time, the tryptophan (Trp) or tyrosine (Tyr) content was followed by monitoring the characteristic Trp or Tyr fluorescence. In further experiments, the degree of protein oxidation was monitored spectrophotometrically by derivatization with 2,4-dinitrophenylhydrazine (DNPH) as described in [77].

5.2.11. Lipid peroxidation

For these experiments, Jurkat cells were seeded at the concentration of 50.000 cells/mL in 24-well tissue culture microtiter plates and then were irradiated in HBSS in the presence of the test

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compounds. Lipid peroxidation was assessed following the method of Morliere et al. [74]. A standard curve of 1,1,3,3-tetraethoxypropane was used to quantify the amount of produced malonaldehyde. Data were expressed in terms of nanomoles of TBARS normalized to the total protein content in an aliquot of the cell extract.

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Appendix A. Supplementary data

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