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Pyridazinopsoralens of wide chemotherapeutic interest

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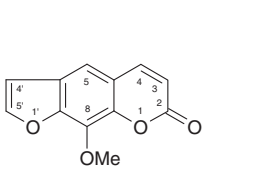
ABSTRACT

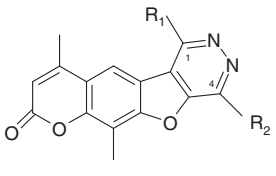
The synthesis of new 6,10-dimethylpyridazino[4,5-*h*]psoralens, carrying no (**4**), one (**5**), or two (**6**–**9**) dialkylaminoalkylcarboxamide side chains on the pyridazine ring is reported. All compounds exert a significant photoantiproliferative activity. Moreover, the derivatives characterised by the protonable side chains show a notable cytotoxicity in the dark. The investigation on the mechanism of action demonstrated the capacity to intercalate into DNA base pairs and to inhibit the relaxation activity of topoisomerase II.

1. Introduction

The interest in psoralens, especially 8-methoxypsoralen (8-MOP, Fig. 1) and 5-methoxypsoralen (5-MOP), comes from their effectiveness in PUVA (psoralen plus UVA) therapy for the treatment of psoriasis and a number of other skin diseases, and of cutaneous T-cell lymphoma.[1–4] The planar aromatic structure, consisting of a furan ring fused to a coumarin moiety, and hydrophobic nature facilitate the intercalation of psoralens between DNA base pairs in the ground state; this intercalation produces the so-called dark complex. Nevertheless, the cellular effects are mostly correlated with the photoreaction of the tricyclic chromophore with DNA bases. The photoaddition involves the 4′,5′ and/or 3,4 double bond of the psoralen and the 5,6 double bond of a pyrimidine base, mainly thymine.[5] However, the occurrence of both short- (erythema, hyperpigmentation) and long-term (benign keratoses, premalignant keratoses, skin cancer) side effects stimulated the design and synthesis of new psoralen analogues. Interestingly, in this connection a number of tetracyclic derivatives, resulting from the condensation of a fourth benzenic or cyclohexenyl ring at the 4′,5′ or 3,4 double bond of the psoralen moiety, showed very interesting photobiological properties.[6–8] In particular, the tetracyclic analogues carrying a protonable dimethylaminopropoxy side chain showed a reduction of skin phototoxicity, which was determined by evaluating the appearance of cutaneous sensitisation on guinea pigs, and a notable increase in the photoantiproliferative activity with respect to 8-MOP. Moreover, unlike the reference drug, the most active compounds showed a significant antiproliferative effect in the dark, that is, independently from UVA irradiation. This latter effect was attributed to an effective complexation with DNA through an intercalative mode of binding in the ground state, and indeed for some derivatives the capacity to interfere with the catalytic activity of DNA topoisomerase II was demonstrated.[6,8] More recently, we synthesised and studied a tetracyclic psoralen derivative having a fourth pyridazine ring condensed at the 4′,5′ photoreactive double bond of the psoralen chromophore and carrying a dimethylaminopropoxy side chain.[9] In accordance with previous results, the above pyridazinopsoralen



8-MOP



4: $R_1 = R_2 = H$
5: $R_1 = CONH(CH_2)_3N(CH_2CH_3)_2$; $R_2 = H$
6: $R_1 = R_2 = CONH(CH_2)_3N(CH_2CH_3)_2$
7: $R_1 = R_2 = CONH(CH_2)_3N(CH_3)_2$
8: $R_1 = R_2 = CONH(CH_2)_2N(CH_2CH_3)_2$
9: $R_1 = R_2 = CONH(CH_2)_2N(CH_3)_2$

Figure 1. Chemical structure of 8-methoxypsoralen (8-MOP) and new pyridazinopsoralens **4**–**9**.

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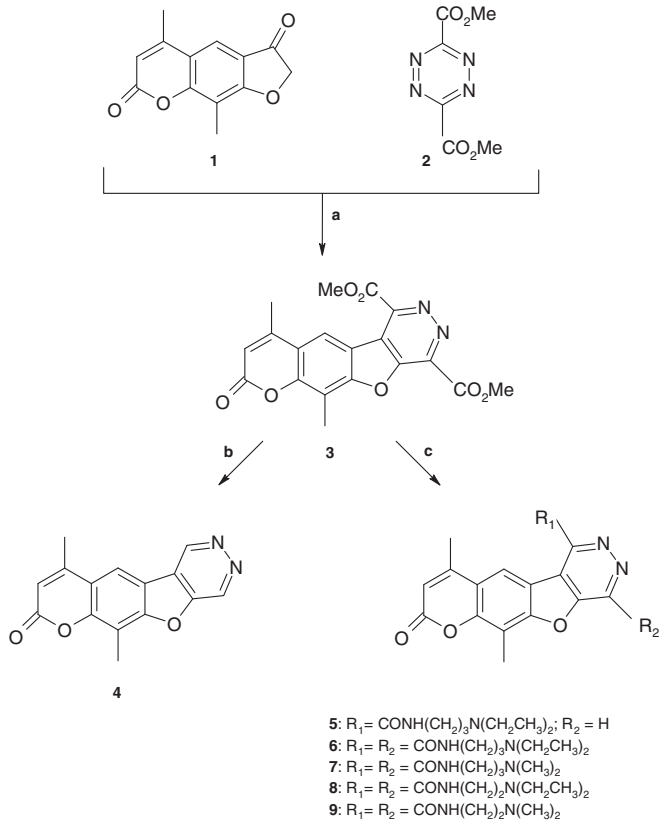
showed a significant reduction in skin phototoxicity, a photoantiproliferative effect higher than that of 8-MOP, and the occurrence of cytotoxic activity even in the dark.[9] Nevertheless, whilst for the benzo and tetrahydrobenzo derivatives the antiproliferative effect shown in the dark appeared notably lower with respect to that exerted upon UVA irradiation,[6–8] for the pyridazinopsoralen the contribution of the dark effect appeared more significant.[9] This behaviour was attributed to a very efficient intercalative capacity between DNA base pairs, likely due to the pyridazine ring. In particular, the wider planarity of the chromophore, along with the tendency to the protonation of the nitrogens of the pyridazine ring and of the side chain, strongly favours an effective complexation.[9] The overall properties of the new pyridazinopsoralen suggested the design of a molecular psoralen-based structure endowed with anticancer effect. Such molecular structure could wide the therapeutic applications of PUVA therapy; this overcomes the limit constituted by the poor penetration ability of the short wavelengths employed (320–400 nm). Indeed, the concurrent dark cytotoxic effect allows an effective treatment not only for skin hyperproliferative diseases, but also for superficial bladder or aerodigestive tract cancer.

On the basis of these considerations, we have synthesised and studied a series of pyridazino[4,5-*h*]psoralens (**4**–**9**) (Fig. 1). The new derivatives are characterised by the insertion of one (**5**) or two (**6**–**9**) dialkylaminoalkylcarboxamide side chains in the positions 1 and 4 of the pyridazine ring. The antiproliferative activity both in the presence of UVA (365 nm) light and in its absence was evaluated. The capacity to form a noncovalent molecular complex with DNA was studied by spectroscopic (linear flow dichroism analysis) and electrophoretic (unwinding assay) techniques. Finally, the ability to interfere with the catalytic cycle of the nuclear enzyme topoisomerase II was investigated.



Scheme 1. Reagents and conditions: (a) *p*-TsOH, CH_2Cl_2, 100 °C; (b) 1:1 AcOH/HCl, reflux; (c) diamine, $MgCl_2$, rt.

2. Results and discussion

2.1. Chemistry

The synthesis of the studied compounds was performed as depicted in Scheme 1. Attachment of a pyridazine ring to a furan nucleus through a Diels–Alder reaction with a tetrazine[10] requires the dienophile to be a furan with a good leaving group in position 3; otherwise ring opening occurs rather than aromatisation of the resulting pyridazine.[11] Therefore, we chose to synthesise the desired pyridazinofurocoumarin **3** from the appropriate precursor furocoumarin-6-one by reaction with 3,6-bis(methoxycarbonil)-1,2,4,5-tetrazine[12] in dichloromethane: this mixture was heated to 100 °C in a sealed tube in the presence of *p*-toluenesulfonic acid, which yielded 74%.[13,14] The synthesis of the final carboxamides was performed with good yields by amination reaction in dichloromethane and presence of magnesium chloride at room temperature for approximately 3 h.[15] The formation of monoamide **5** occurs in the reaction of formation of diamide **6** after 6 h of reaction. The two carboxymethyl groups present on this tetracyclic skeleton could be eliminated by refluxing with a mixture of 1:1 acetic acid and 37% hydrochloric acid to obtain the pyridazinofurocoumarin **4** in 75% yield.[14]

2.2. Antiproliferative activity

The antiproliferative activity of new pyridazinopsoralens **4**–**9** was evaluated by means of an in vitro assay performed on human tumour cell lines. The results, expressed as IC_{50} values, that is, the concentration (μM) of compound able to induce 50% cell death with respect to a control culture, are shown in Table 1.

The presence of the 3,4 photoreactive double bond at pyrone side of the psoralen nucleus prompts us to investigate the photoantiproliferative effect of these derivatives. The experiments performed on HL-60 cell line in the presence of UVA light (365 nm, 0.789 J cm^{-2}) showed for the new pyridazinopsoralens a significant activity, up to seven times higher with respect to that exerted by 8-MOP, taken as reference drug. Moreover, interestingly, the occurrence of skin phototoxicity, evaluated as appearance of erythema on depilated guinea pigs,[8] demonstrated for the new compounds a significantly lower cutaneous photosensitization, with respect to 8-MOP. In fact, the drug, when applied topically at the same concentration, induces a strong erythema with oedema (data not shown). Taking into consideration that erythema constitutes the most common short-term side effect in PUVA therapy, the above results indicate the new pyridazinopsoralen structure as an interesting pattern inside the photobiological field. The incubation of HL-60 cells for 24 h in the dark (in the absence of UVA light), confirmed for 8-MOP the inability to induce any cytotoxicity, whilst for **4**–**9** showed very interesting results. Indeed, for the new pyridazinopsoralens an antiproliferative effect similar (compounds **4**–**7**) or even higher (compounds **8** and **9**) to that obtained

Table 1
HL-60 cell growth inhibition in the presence of examined compounds and 8-MOP as reference drug

Compound	IC_{50} (μM)		
	UVA	Dark 24 h	Dark 72 h
4	4.1 ± 0.3	3.9 ± 0.4	2.6 ± 0.7
5	2.2 ± 0.2	2.1 ± 0.1	0.9 ± 0.1
6	0.8 ± 0.1	0.9 ± 0.1	0.37 ± 0.1
7	2.6 ± 0.3	3.8 ± 0.3	0.61 ± 0.1
8	3.5 ± 0.4	1.2 ± 0.08	0.57 ± 0.06
9	1.3 ± 0.3	0.9 ± 0.1	0.41 ± 0.1
8-MOP	5.4 ± 0.7	>20	>20

upon UVA irradiation was achieved. To evaluate the UVA contribution, the cell growth inhibition assay was performed by using the adherent HeLa cells, which allow to remove the test compound just after the irradiation or after 60 min in the dark (see Section 4). The antiproliferative activity on cells exposed to UVA light is higher, with IC_{50} values from 1.5 to 4.9 times lower than those found in the dark, demonstrating the occurrence of some photoantiproliferative effect.

Altogether, the above results point to the new tetracyclic pyridazine chromophore as a chemical structure endowed with significant antiproliferative properties apart from UVA irradiation. To assess this assumption, the cells were incubated in the presence of the new derivatives **4**–**9** for 72 h, according to a well established procedure.[16] Interestingly, the IC_{50} values of **5**–**9**, which are characterised by the insertion of one (**5**) or two (**6**–**9**) dialkylaminoalkylcarboxamide side chains, are from two to six times lower with respect to those obtained by incubating the cells for 24 h. For the unsubstituted chromophore (compound **4**), the IC_{50} is practically unchanged. Moreover, a comparison between the antiproliferative activity of **4**, that is, the unsubstituted chromophore, and the corresponding derivatives carrying one (compound **5**) or two (compound **6**) side chains, shows that the cytotoxic capacity clearly increases from **4** to **6**. Altogether, these results indicate the pyridazinopsoralen nucleus as a chemical structure endowed with anticancer properties, and further suggest a crucial role for the protonable side chains.

2.3. Interaction with salmon testes DNA

The capacity of the psoralens and tetracyclic psoralen derivatives to intercalate between DNA base pairs has already been demonstrated[6–8] and, more recently, this ability was also confirmed for a tetracyclic pyridazinopsoralen chromophore.[9] To investigate if the new derivatives **4**–**9** are also capable to form a molecular complex with the macromolecule, linear flow dichroism (LD) experiments were performed. Figure 2A–C shows, as an example, the spectra of DNA solution in the presence of different concentrations of **4**–**6**, respectively. In DNA spectra (traces a) the occurrence of the typical negative signal at 260 nm, attributable to the base pairs absorption, can be observed. In the presence of test compounds (traces b–d), a further dose-dependent negative signal appears at higher wavelengths (300–380 nm), which is the spectral region where only the pyridazinopsoralen chromophore absorbs. Similar behaviours were also obtained for **7**–**9** (spectra not shown). The appearance of this latter negative signal is due to an orientation of the tetracyclic chromophore, which can occur as a consequence of the complexation with the macromolecule. Moreover, the negative sign of the LD signal is in accordance with an orientation of the pyridazinopsoralen molecular plane, which is preferentially parallel to the plane of DNA bases.[17] These results also clearly demonstrated for the new pyridazinopsoralen derivatives **4**–**9** the ability to form a complex with the macromolecule, through an intercalative mode of binding.

2.4. Unwinding assay with supercoiled pBR322 DNA

In an attempt to further investigate the complexation ability of the structurally related **4**–**6** with the nucleic acid, an unwinding assay with supercoiled plasmid pBR322 DNA was also performed. In detail, an increase in concentration of an intercalating agent induces a slowing in the intrinsic electrophoretic mobility of supercoiled DNA, which is due to the unwinding process that leads to the supercoiled form. A further increase in concentration provokes a reversion to the original electrophoretic rate, as a consequence of a supercoiling process that restores the circular form. The effect of increasing concentrations of **4**–**6** on pBR322 is shown in Figure 3A–C, respectively. In the presence of the unsubstituted pyridazinopsoralen **4** no detectable effect can be revealed on plasmid DNA up to 200 μM concentration (Fig. 3A). On the contrary, by incubating pBR322 with increasing concentration of **5**, a complete titration curve is obtained with a minimum of migration rate around 50 μM (Fig. 3B). A more marked slowing of migration rate is observed when supercoiled DNA is incubated in the presence of the derivative **6**, carrying both dialkylaminoalkylcarboxamide side chains, and indeed a complete reversion of the supercoiled form to the circular one already appears at 5 μM concentration (Fig. 3C). The obtained results suggest a different complexation ability for the three compounds, and in particular an increasing intercalative capacity from **4** to **6**. This behaviour is in agreement with the antiproliferative data (Table 1) and suggests that the capacity to form

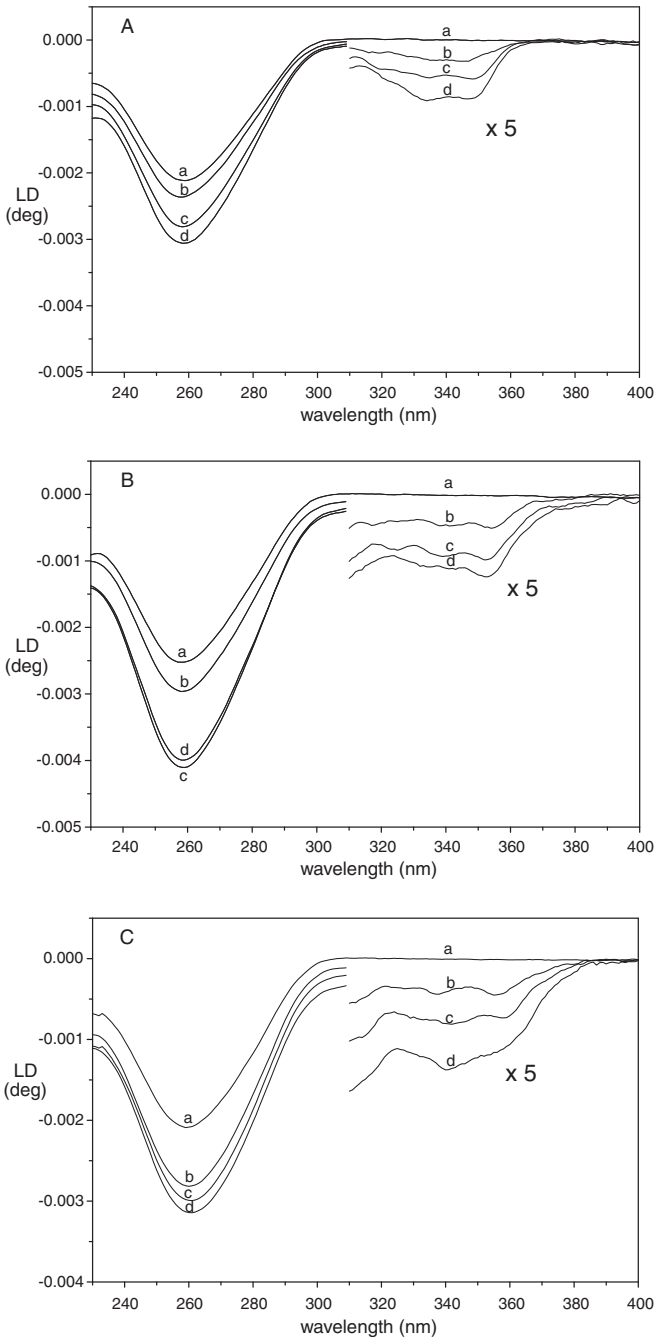


Figure 2. Linear flow dichroism for compounds **4** (A), **5** (B) and **6** (C) at different [drug/DNA] ratios: line a, 0; b, 0.02; c, 0.04; d, 0.08. [DNA] = 1.6×10^{-3} M.

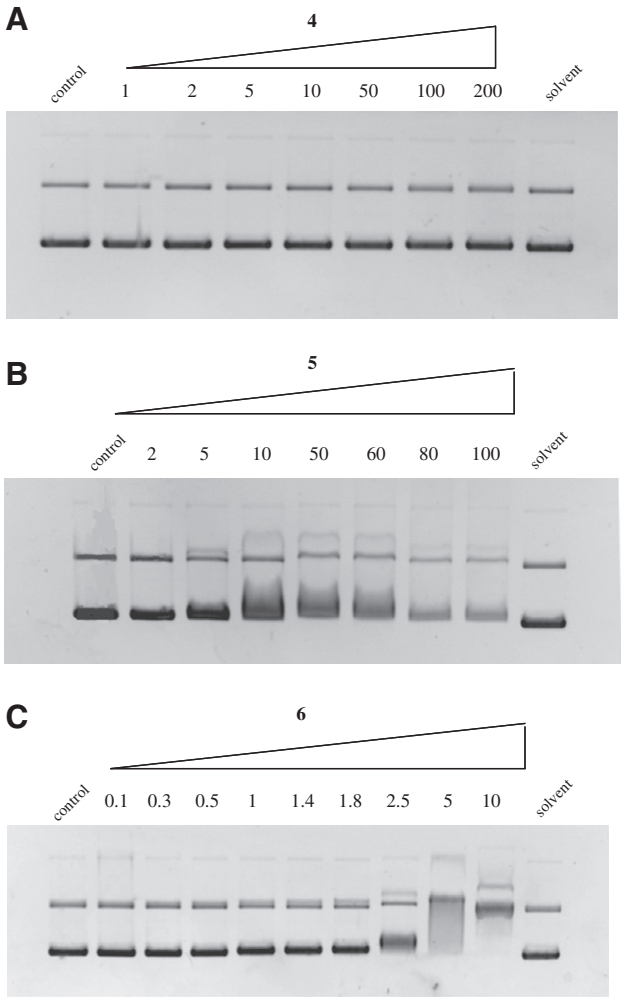


Figure 3. Effect of compounds **4** (A), **5** (B) and **6** (C) on the supercoiling of pBR322 DNA (control) at indicated concentrations (μM).

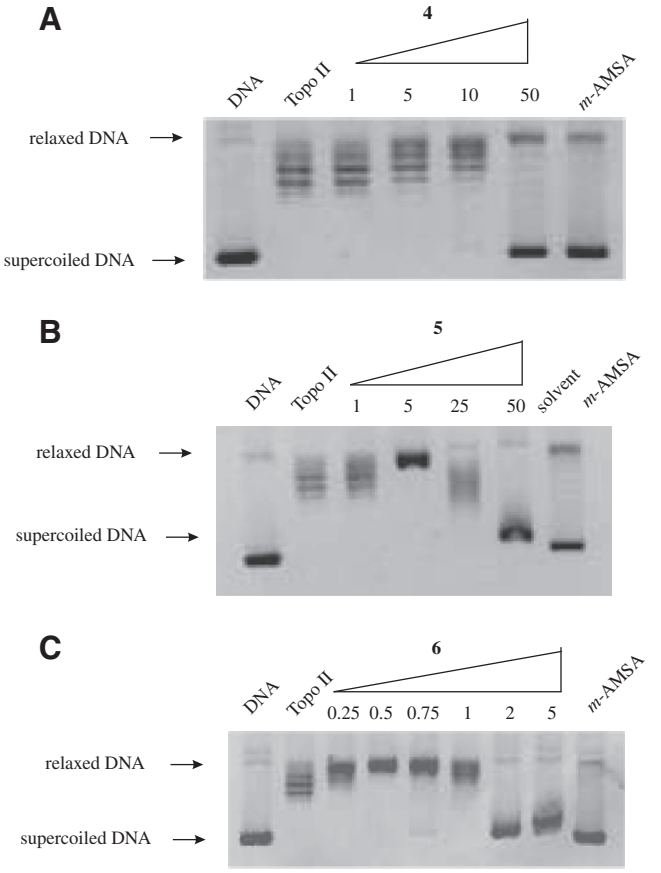


Figure 4. Effect on relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with topoisomerase II in the absence (Topo II) and presence of compounds **4** (A), **5** (B) and **6** (C) at indicated concentration (μM); 8 μM *m*-AMSA was used as reference.

a molecular complex with the macromolecule participates in the mechanism of action of these pyridazinopsoralen structures.

2.5. Effect on topoisomerase II activity

DNA topoisomerase II is a nuclear enzyme that solves the topological problems that arise in DNA double strand during transcription, replication and other genomic processes.[18,19] The key role played by the enzyme in cell proliferation rendered it an intriguing target for the anticancer strategy and indeed, many active anticancer drugs affect its catalytic activity. In particular, a number of intercalating drugs, including anthracyclines, mitoxantrone and *m*-AMSA, target DNA topoisomerase II.[20] In this connection, the capacity of **4**–**6** to intercalate into base pairs, suggested that their cytotoxicity could be related to an interference with topoisomerase II activity. Figure 4A–C shows the effect of increasing concentrations of the new pyridazinopsoralens **4**–**6** on the relaxation, mediated by topoisomerase II, of supercoiled plasmid pBR322 DNA. The effect of the drug *m*-AMSA was reported as reference. The appearance of supercoiled DNA in the presence of test compound, along with a corresponding decrease in the relaxed form, is indicative of an inhibition of the enzymatic activity. For all new pyridazinopsoralens **4**–**6** a dose-dependent inhibitory effect on relaxation is obtained and interestingly, it appears in accordance with both the intercalative (Fig. 3A–C) and antiproliferative capacity (Table 1). In particular, the unsubstituted **4**, which exerts the lower cytotoxicity, at 50 μM provokes a significant but not complete block of topoisomerase II activity (Fig. 4A). On the other hand, **5**, which shows an IC_{50} value more than two times lower, at the same concentration completely inhibits the enzymatic activity (Fig. 4B). Finally, compound **6**, characterised by the insertion of two dialkylaminoalkylcarboxamide side chains, and by the higher intercalative capacity and cytotoxicity, completely inhibits the relaxation activity at 2 μM (Fig. 4C).

Drugs targeting topoisomerase II can be divided into two groups: poisons and catalytic inhibitors. The poisons stabilize the covalent DNA topoisomerase II complex, thus generating DNA strand breaks, and include most of the clinically active drugs. The catalytic inhibitors act on any of the other steps of the catalytic cycle and are thought to kill cells through the elimination of the essential enzymatic activity of topoisomerase II.[20,21] To discriminate whether the most active compound **6** acts as catalytic inhibitor or poison, a cleavage assay was performed and the DNA forms are resolved on agarose gel containing ethidium bromide, as previously reported.[16] In these conditions the relaxed DNA migrates faster than the supercoiled plasmid and the occurrence of the cleavage complex can be demonstrated by the enzyme-dependent formation of linear DNA (Fig. 5). *m*-AMSA, a well-known topoisomerase II poison, was tested as reference compound. The results show that the pyridazine derivative up to 50 μM concentration does not promote DNA cleavage by topoisomerase II because the intensity of the band corresponding to linear DNA is not amplified. Otherwise, in the presence of 8 μM *m*-AMSA, a known topoisomerase II poison,[20] the appearance of a significant amount of linear DNA is observed.

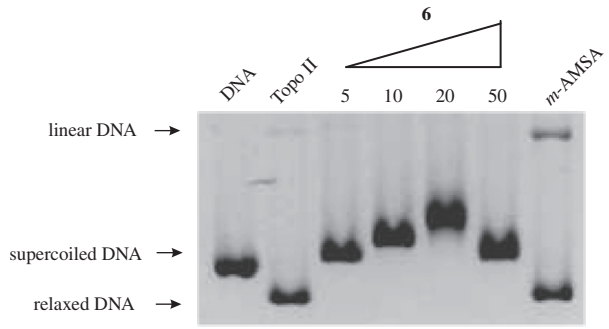


Figure 5. Effect of compound **6** on the stabilization of covalent DNA topoisomerase II complex. Supercoiled DNA (DNA) was incubated with topoisomerase II in the absence (Topo II) and presence of compound **6** at indicated concentration (μM); 8 μM *m*-AMSA was used as reference.

The above results demonstrate for **6** the inability to stabilize the cleavage complex and suggest that the inhibition of the topoisomerase II-mediated relaxation could be a consequence of DNA intercalation. Therefore, **6** is not a topoisomerase II poison, but could be considered a catalytic topoisomerase II inhibitor.

3. Conclusions

The synthesis of new 6,10-dimethylpyridazino[4,5-*h*]psoralens (**4**–**9**) carrying one (**5**) or two (**6**–**9**) dialkylaminoalkylcarboxamide side chains was performed. The study of the photobiological properties pointed out for the new derivatives a photoantiproliferative effect comparable, and in some cases better, than that of 8-MOP. In addition, the new pyridazinopsoralens exerted a significant cytotoxicity in the absence of UVA irradiation, which increases by extending the incubation time, thus demonstrating an interesting antitumor ability. Regarding the mechanism of action, the new compounds form an intercalative complex with DNA and interfere with the enzymatic activity of DNA topoisomerase II. The concurrent ability to induce a UVA-dependent and independent antiproliferative effect could enlarge the therapeutic effectiveness of PUVA therapy. This new property renders the pyridazinopsoralen structure a noteworthy model for the development of novel antitumor drugs.

4. Experimental

4.1. General

Melting points are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. IR spectra were recorded with a Perkin-Elmer 1640FT spectrometer (KBr disks, υ in cm^{-1}). ^1H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra of the synthetic compounds were recorded with a Bruker AMX spectrometer, using TMS as internal standard (chemical shifts in δ values, *J* in Hz). Mass spectrometry was carried out on a Hewlett-Packard 5988A or on a Finnigan Trace MS spectrometer. Elemental analyses were performed with a Perkin-Elmer 240B microanalyzer and were within (0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254, 0.25 mm).

4.1.1. 6,10-Dimethylpyridazino[4,5-*h*]psoralen (4)

A solution of the diester (**3**, 54 mg, 1.14 mmol) in 1:1 AcOH/HCl (20 mL) was refluxed for 3 h. The solvent was evaporated under reduced pressure. A saturated solution of $NaHCO_3$ (35 mL) was added to the residue and the precipitate was recovered by filtration and washed with water. The white solid was overnight dried and then purified by FC using 95:5 CH_2Cl_2/MeOH as eluent, given pure **4** (26 mg, 75%): mp 310–312 °C. IR (KBr) 1715 (CO), 1636, 1334, 1093 cm^{-1}. ^1H RMN (DMSO-d_6) δ 10.13 (d, J = 1.2 Hz, 1H), 9.94 (d, J = 1.2 Hz, 1H), 8.70 (s, 1H, H_{11}), 6.50 (d, J = 1.1 Hz, 1H, H_9), 2.59 (s, 3H), 2.57 (d, J = 1.1 Hz, 3H). ^{13}C RMN (DMSO-d_6): δ = 160.10 (CO), 156.50, 155.01, 154.55 (CH), 153.77, 146.07, 139.07, 122.15 (CH), 117.94, 117.90 (CH), 115.53, 113.75 (CH), 110.26 (CH), 19.01, 8.93. EM (CI): *m/z* (%) = 266.9 (M^{+1}, 23). HRMS-CI^+ calcd for $C_{15}H_{11}N_2O_3$: 267.0770. Found: 267.0760.

4.2. Preparation of the carboxamides (5–9): general procedure

To the dimethoxycarbonylpyridazinofurocoumarin (**3**,[13] 1 mmol) in dry CH_2Cl_2 (10 mL) was added anhydrous $MgCl_2$ (3 mmol) and the mixture was stirred for 5 min. at room temperature. *N*,*N*-Dimethylethylenediamine (5 mmol) was added and the mixture was stirred for 3–6 h at room temperature. The mixture was acidified with 0.5 M HCl (15 mL) and washed with CH_2Cl_2. The aqueous phase was then basified with $NaHCO_3$ and extracted with CH_2Cl_2. The organic phase was washed, dried over $NaSO_4$, evaporated under reduced pressure, and purified by flash chromatography (SiO_2) using CH_2Cl_2/EtOH/NH_3 (68:30:2) as eluent.

Diamine	Product (yield)
$H_2N(CH_2)_3N(CH_2CH_3)_2$	**5** (21%) and **6** (38%)
$H_2N(CH_2)_3N(CH_3)_2$	**7** (72%)
$H_2N(CH_2)_2N(CH_2CH_3)_2$	**8** (40%)
$H_2N(CH_2)_2N(CH_3)_2$	**9** (74%)[13]

4.2.1. 6,10-Dimethyl-1-(diethylaminopropylcarbamoyl)pyridazino[4,5-*h*]psoralen (5)

Mp: 189–191 °C IR (KBr): 3340 (NH), 1721 (CO–O), 1650 (CO–N), 1443, 1364, 1261, 1218, 1179, 1104 cm^{-1}. ^1H MNR ($CDCl_3$) δ 9.70 (s, 1H, H_4), 9.53 (s, 1H, NH), 9.49 (s, 1H, H_{11}), 6.32 (s, 1H, H_9), 3.71 (m, 4H), 2.72 (s, 3H), 2.68 (s, 3H), 2.60 (m, 12H), 1.84 (m, 4H), 1.05 (t, 12H). ^{13}C MNR ($CDCl_3$) δ 163.34 (CO), 160.15 (CO), 157.28, 156.26, 153.64, 153.47, 148.25, 139.14, 123.25, 122.54, 118.06, 115.31, 113.90, 110.30, 51.83, 46.81, 39.36, 26.11, 19.50, 11.44, 8.80. HRMS-CI^+ calcd for $C_{23}H_{28}N_4O_4$: 424.2111. Found: 424.1837.

4.2.2. 6,10-Dimethyl-1,4-bis(diethylaminopropilcarbamoyl)-pyridazino[4,5-*h*]psoralen (6)

Mp: 240–242 °C. IR (KBr) 3321 (NH), 1726 (CO–O), 1666 (CO–N), 1638 (CO–N), 1596, 1560, 1526,1443, 1364, 1261, 1218, 1179, 1104 cm^{-1}. ^1H MNR ($CDCl_3$) δ 9.63 (s, 1H, NH), 9.44 (s, 1H, NH), 9.37 (s, 1H, H_{11}), 6.26 (s, 1H, H_9), 3.66 (m, 4H), 2.68 (s, 3H), 2.61 (s, 3H), 2.52 (m, 12H), 1.79 (m, 4H), 1.02 (t, 12H). ^{13}C MNR ($CDCl_3$) δ 162.80 (CO), 160.98 (CO), 159.93 (CO), 157.93, 155.17, 153.71, 153.26, 149.17, 140.42, 124.72, 123.02, 117.96, 114.62, 113.82, 110.65, 52.13, 46.92, 39.88, 39.68, 26.50, 25.96, 19.56, 11.72, 9.07. HRMS-CI^+ calcd for $C_{31}H_{44}N_6O_5$: 580.3373. Found: 580.2875.

4.2.3. 6,10-Dimethyl-1,4-bis(dimethylaminopropilcarbamoyl)-pyridazino[4,5-*h*]psoralen (7)

Mp: 253–256 °C. IR (KBr) 3324 (NH), 1725 (CO–O), 1665 (CO–N), 1627 (CO–N), 1595,1524,1442, 1362, 1333, 1216, 1178, 1103 cm^{-1}. ^1H MNR ($CDCl_3$) δ 9.30 (s, 1H, H_{11}), 9.24 (s, 1H, NH), 9.04 (s, 1H, NH), 6.28 (s, 1H, H_9), 3.72 (m, 4H), 2.67 (s, 3H), 2.60 (s, 3H), 2.48 (m, 4H), 2.30 (s, 6H), 2.28 (s, 6H), 1.88 (m, 4H). ^{13}C MNR ($CDCl_3$) δ 162.54 (CO), 160.75 (CO), 159.72 (CO), 157.78, 155.38, 154.02, 153.65, 153.11, 148.94, 140.14, 124.69, 122.87, 117.90, 114.32, 113.80, 110.58, 58.06, 58.03, 45.51, 45.49, 39.08, 26.7, 19.45, 8.96. HRMS-CI^+ calcd for $C_{27}H_{36}N_6O_5$: 524.2747. Found: 524.2303.

4.2.4. 6,10-Dimethyl-1,4-bis(diethylaminoethylcarbamoyl)-pyridazino[4,5-*h*]psoralen (8)

Mp: 222–225 °C. IR (KBr) 3317 (NH), 1726 (CO–O), 1667 (CO–N), 1631 (CO–N), 1596, 1520, 1444, 1364, 1334, 1300, 1216, 1179, 1103 cm^{-1}. ^1H MNR ($CDCl_3$) δ 9.33 (s, 1H, H_{11}), 8.77(s, 1H, NH), 8.52 (s, 1H, NH), 6.30 (s, 1H, H_9), 3.66 (m, 4H), 2.76 (s, 3H), 2.71 (s, 3H), 2.62 (m, 12H), 1.07 (t, 12H). ^{13}C MNR ($CDCl_3$) δ 162.52 (CO), 160.69 (CO), 160.00 (CO), 159.70, 157.85, 155.09, 153.74, 153.09, 148.94, 140.11, 124.85, 122.92, 118.00, 114.35, 113.88, 110.66, 51.46, 47.00, 46.62, 37.63, 37.40, 29.70, 19.48, 11.93, 9.01. HRMS-CI$^+$ calcd for $C_{29}H_{40}N_6O_5$: 552.3060. Found: 552.2894.

4.3. Cell cultures

HL-60 and HeLa cells were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated foetal calf serum (Biological Industries) and Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated foetal calf serum (Biological Industries), respectively. Penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) (Sigma Chemical Co.) were added to the medium. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

4.4. Irradiation procedure

Irradiations were performed by means of Philips HPW 125 lamps equipped with a Philips filter emitting over 90% at 365 nm. Irradiation intensity was checked on a UV-X radiometer (Ultraviolet Products Inc., Cambridge, UK) for each experimental procedure.

4.5. Inhibition growth assays

HeLa cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) without phenol red, and various concentrations of the test agent were added. One hour later the cells were irradiated with a UVA dose of 0.789 J cm^{-2}. After irradiation, the medium containing the compounds was removed, and the cells were incubated in complete F-12 medium for 24 h. For the experiments carried out in the dark, after incubation for 24 h, various concentrations of the test agent were added, 1 h later the medium containing the compounds was removed, and the cells were incubated in complete F-12 medium for 24 h.

HL-60 cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of test agents were added in complete medium. One hour later the cells were irradiated with a UVA dose of 0.789 J cm^{-2}, and then incubated for further 24 h.

In the case of the experiments carried out in the dark, HL-60 cells (10^5 or 4×10^4) were seeded into each well of a 24-well cell culture plate; after incubation for 24 h, various concentrations of the test compounds were added in complete medium and incubated for further 24 or 72 h, respectively.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data are expressed as IC_{50} values, that is, the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

4.6. Nucleic acids

Salmon testes DNA was purchased from Sigma Chemical Company (Cat. D-1626). Its hypocromicity, determined according to Marmur and Doty,[22] was over 35%. The DNA concentration was determined using extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm. pBR322 DNA was purchased from Fermentas Life Sciences.

4.7. Linear flow dichroism

Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter, converted for LD and equipped with an IBM PC and a Jasco J interface. Linear dichroism was defined as:

$$LD_{(\lambda)} = A_{\parallel(\lambda)} - A_{\perp(\lambda)}$$

where $A_\parallel$ and $A_\perp$ correspond to the absorbances of the sample when polarised light was oriented parallel or perpendicular to the flow direction, respectively. The orientation was produced by a device designed by Wada and Kozawa[23] at a shear gradient of 500–700 rpm, and each spectrum was accumulated twice.

Aqueous solutions of DNA (1.9×10^{-3} M) in 10 mM TRIS, 1 mM EDTA (pH 7.0) and 0.01 M NaCl were used (ETN buffer). Spectra were recorded at 25 °C at [drug]/[DNA] = 0, 0.02, 0.04 and 0.08.

4.8. Unwinding assay

Solutions of tested drugs were prepared at the required concentrations; 0.5 μL of solution was mixed with 150 ng of supercoiled pBR322 DNA in TAE buffer (40 mM TRIS, 20 mM glacial acetic acid and 1 mM EDTA, pH 8) to reach a final reaction volume of 10 μL. Complexes were incubated in the dark for 30 min at 37 °C. Following incubation, 3 μL of loading buffer (50% glycerol and 10% bromophenol blue) was added, and the samples were loaded into a 0.8% agarose gel. Electrophoresis was continued for 1 h at 74 V at room temperature. After electrophoresis, the gel was stained for 30 min in a TAE bath for an additional 20 min. The gel was transilluminated by UV light and fluorescence emission visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.9. Topoisomerase II-mediated DNA relaxation

Supercoiled pBR322 plasmid DNA (0.25 μg) was incubated with 1 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 μL reaction buffer.

Reactions were stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide 1 μg/mL in TAE buffer, transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.10. Topoisomerase II-mediated DNA cleavage

Supercoiled pBR322 plasmid DNA (0.25 μg) was incubated with 10 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 μL reaction buffer.

Reactions were stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL in TBE buffer (0.09 M Tris–borate and 0.002 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.006.

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