ELSEVIER

Il Farmaco 53 (1998) 494–503

IL FARMACO

Pyran derivatives XX. 2-Aminochromone benzo-fused derivatives with antiproliferative properties

Giorgio Roma [a,*], Mario Di Braccio [a], Giancarlo Grossi [a], Cristina Marzano [b], Morena Simonato [b], Franco Bordin [b]

[a] *Dipartimento di Scienze Farmaceutiche dell'Università, Viale Benedetto XV 3, 16132 Genoa, Italy*

[b] *Dipartimento di Scienze Farmaceutiche dell'Università, Centro di Studio sulla Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR (associated with the Istituto Nazionale per la Chimica dei Sistemi Biologici), 35100 Padua, Italy*

Received 5 May 1998; accepted 3 June 1998

Abstract

The *N*-substituted 2-aminochromones **1** and their benzo-fused derivatives **2–4** described herein were mostly prepared by treating the corresponding (methylthio) derivatives **10–13** with an excess of the proper amines. Only the morpholino derivatives **3d** and **4c** were obtained from the reaction of the ethyl 3-morpholino-3-oxopropanoate/$POCl_3$ reagent with 1-naphthol or 1-methyl-2-naphthol, respectively. The amino derivatives **1–4**, as well as their methylthio analogues **10–13**, were tested in vitro for their inhibitory activity on the infectivity of T2 bacteriophage, on the macromolecular synthesis in Ehrlich cells and on the clonal growth capacity of HeLa cells. Several of the angular or linear aminonaphthopyranones **2** and **3** or **4**, respectively, and the (methylthio) derivatives **10**, **11** and **13** induced a significant inhibition of DNA synthesis, but usually a clearly lower inhibition of clonal growth. Only the linear 2-amino-10-methyl-4*H*-naphtho[2,3-b]pyran-4-ones **4a** and **4b** significantly inhibited the clonal growth in HeLa cells and T2 bacteriophage infectivity, respectively.

Keywords: Benzo-fused 2-aminochromones; Antiproliferative activity

1. Introduction

In previous papers we described the first examples of 2-(dialkylamino)chromones **1** [1,2] and of their benzo-fused angular and linear derivatives **2** [3,4], **3** [5] and **4** [6] (Fig. 1). These 1-benzopyran and naphthopyran derivatives were obtained through the cyclocondensation of the ethyl *N,N*-dialkylmalonamate/$POCl_3$ Vilsmeier-type reagent with the proper phenol or naphthol (see Scheme 1).

Compounds **1–4** exhibited different notable activities on the central nervous system (CNS), according to the ring system [1,2,5–9]. We also described the platelet antiaggregating properties of a number of compounds **1–3**, some of which showed interesting in vitro activities [10–12].

Then, taking into account the well-known antiproliferative properties of photoactivated linear and angular furocoumarins (psoralens and angelicins) [13] as well as of photoactivated khellin [14], a natural linear furochromone, and considering the structural similarity of these compounds with tricyclic chromone derivatives **2–4**, we tested two examples of compounds **2** and **4**

$$[N(R)(R') = N(CH_3)_2]$$

for their antiproliferative properties. As these (dimethylamino)naphthopyranones appeared to be active independently of the presence of UV–A light (see Section 3), we planned to evaluate the dark antiproliferative and cytotoxic activities of some appropriate series of compounds **1–4**. Some of these compounds were previously described by us, and the others have now been synthesized (see Section 2).

The chemical and biological results of this study are reported in the present paper.

It must be noted that the smooth muscle cell antichemotactic and antiproliferative properties of 2-morpholinochromone [15] and 2-morpholino-8-(3-pyridinylmethoxy)-chromone [16] have recently been reported in the literature.

* Corresponding author.

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PII S0014-827X(98)00055-X

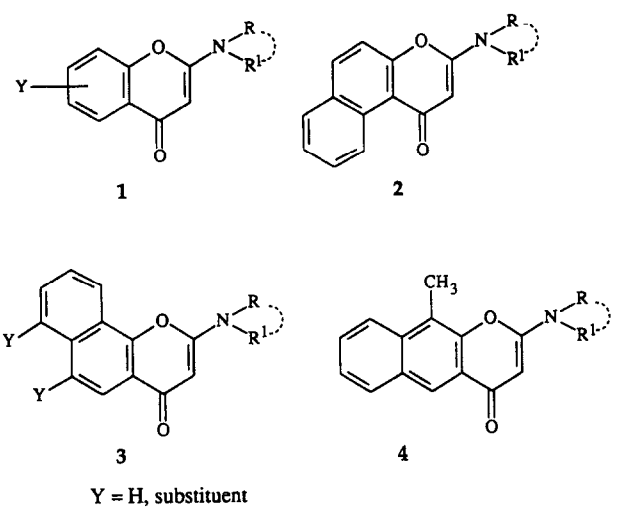


Fig. 1. Structures of the *N*-substituted 2-aminochromones **1** and of their benzo-fused derivatives **2**, **3**, **4**.

In this connection, the specific inhibitory activity of 2-morpholino-8-phenylchromone against phosphatidylinositol 3-kinase has also been described [17].

2. Chemistry

For the preparation of the new chromone derivatives **1a,b**, **2a**, **3a–d** and **4a–c** selected for biological evaluation, two different synthesis methods were employed.

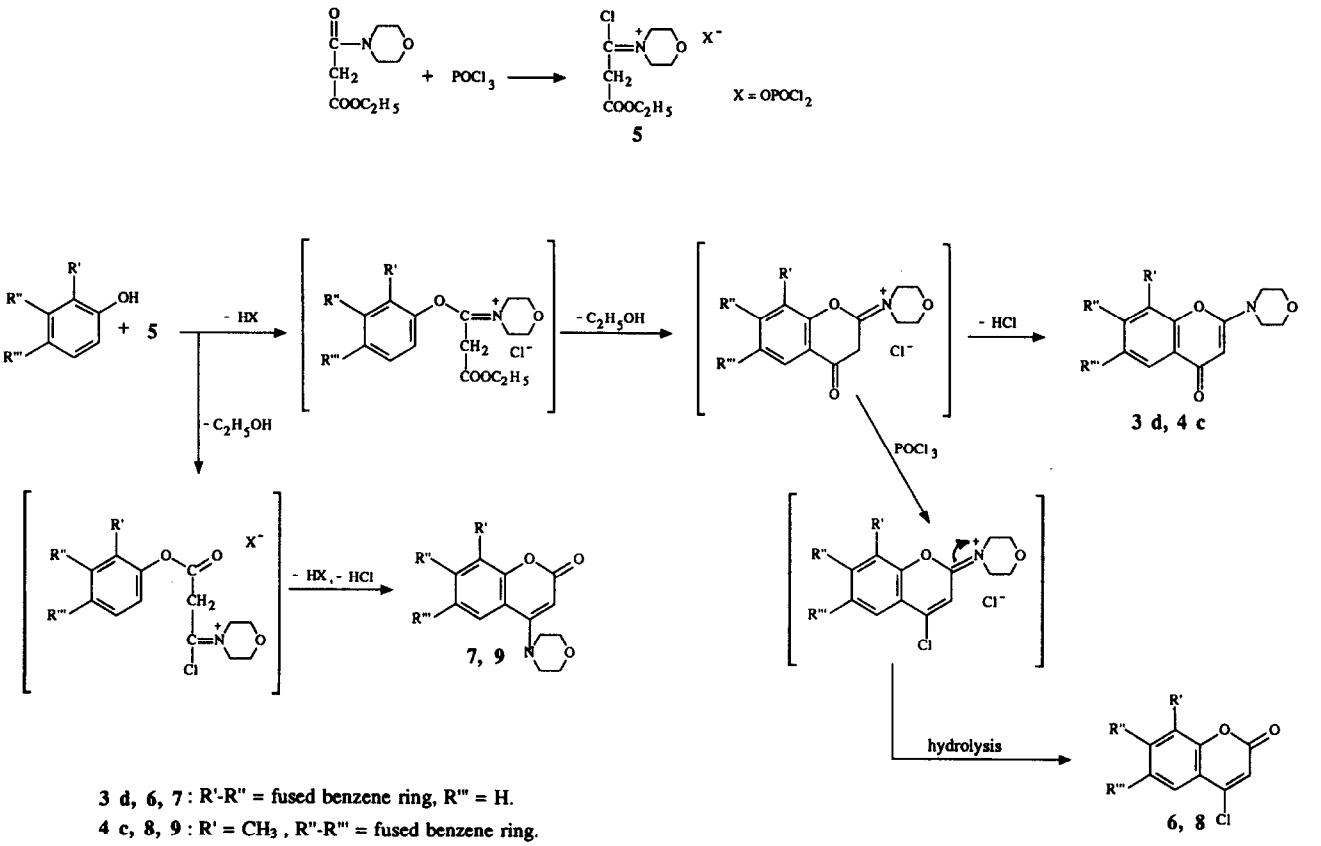
The morpholino derivatives **3d** and **4c** were obtained by the one-pot cyclocondensation (Scheme 1) that we used previously (see above) to synthesize a number of (dialkylamino) derivatives **1–4**, starting from the appropriate commercially available phenols and easily obtainable ethyl *N,N*-dialkylmalonamates.

Thus, the reaction of 1-naphthol with the ethyl 3-morpholino-3-oxopropanoate/$POCl_3$ reagent **5** (chlorobenzene, 130°C, 4 h) afforded a satisfactory yield of compound **3d**, along with its coumarinic isomer **7** and chloro derivative **6** [12]. Starting from 1-methyl-2-naphthol, chromone derivative **4c** (low yield), its isomer **9** and chloro derivative **8** [6] were obtained from the same reaction (chlorobenzene, 110°C, 10 h). In accordance with our previous suggestions [6], the formation of these compounds may be explained through the reaction pattern depicted in Scheme 1.

Since the above cyclocondensation was frequently shown to be unsuitable for giving sufficient yields of the alkylamino substituted compounds **1**, **3** and **4**

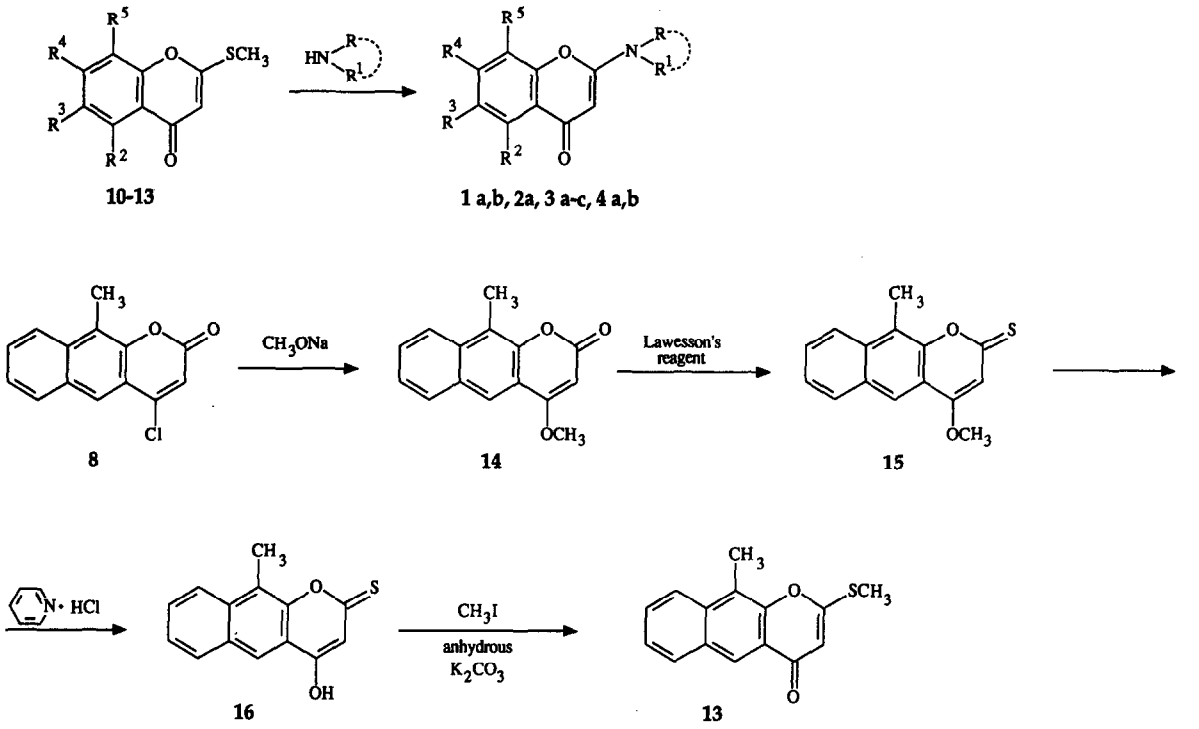
$$\left(N\begin{matrix}R\\R^1\end{matrix} = N\begin{matrix}R\\H\end{matrix}\right)$$

[18], compounds **1a,b**, **2a**, **3a–c** and **4a,b** were all obtained by treating the corresponding (methylthio) derivatives **10–13** with a large excess of the appropriate primary amines (ethylene glycol, 160°C, 1 h; ethanol at reflux, 16 h, only for **3c**) (Scheme 2, Table 1).



3 d, 6, 7: R'-R'' = fused benzene ring, R''' = H.
4 c, 8, 9: R' = CH_3, R''-R''' = fused benzene ring.

Scheme 1.



Scheme 2.

The starting compounds **10–12** were recently prepared by us [12], via the corresponding thiones, through a synthetic method described in the literature [19,20], whereas **13** has now been synthesized as described in Scheme 2. Actually, in this case, the analogue starting compound for the intermediate thione (**16**) was found to be too hard to obtain, and **16** was conveniently prepared from chloro derivative **8**, following a novel synthetic route.

The results of elemental analyses and IR and ^{1}H NMR spectral data were consistent with the structures attributed to the compounds described in this paper (see Section 5 and Table 3).

Thus, as we previously observed in this structural field [1–7,12], the IR spectra (KBr or $CHCl_3$) of the amino or methylthio substituted chromone derivatives now synthesized (**1a,b**, **2a**, **3a–d**, **4a–c**, or **13**) exhibit the ν CO band at characteristic low frequencies (1641–1607 cm^{-1}).

Concerning the ^{1}H NMR spectra of compounds described herein, the chemical shift of the pyran =CH– signal is particularly significant, as we can see by comparing, for instance, the H-3 singlets ($CDCl_3$) of **4c** and **9** (δ 5.50 and 5.85), **4c** and **13** (δ 5.50 and 6.26), **14** and **15** (δ 5.70 and 6.83). Actually, in these examples the difference between the H-3 chemical shifts depends on the chromonic (**4c**) or coumarinic (**9**) structure of the two isomers, or on the different types of 2-substituent (**4c** and **13**, or **14** and **15**). On the other hand, if we compare the H-3 chemical shifts of the 2-aminochromone benzo-fused angular derivatives **3a** (δ 5.90, $CDCl_3$), **3c** (δ 5.40, DMSO-d_6), and **3d** (δ 6.67, CF_3COOD), the influence of the solvent is also evident. With regard to 2-substituted chromones **1a,b** and their benzo-fused analogues **2a**, **4a–c** and **13**, we can further point out the downfield shift of signals of the aromatic protons H-5 (compounds **1**, **4** and **13**) [1,2,6], or H-10 (**2a**) due to the deshielding anisotropy effect of coplanar CO. This effect is very marked in structure **2** [3,4,7] (**2a** ($CDCl_3$): $\delta_{H\text{-}10}$ 10.30). However, the H-10 signals of compounds **3a–d** are shifted more downfield than those of protons H-5 (see Table 3).

Finally, it must be observed that the ^{1}H NMR OH signal of compound **16** (DMSO-d_6) is not clearly detectable, whereas its IR ν OH band (KBr; broad, complex) is present at 3100–2400 cm^{-1}.

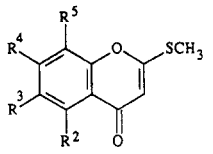
None of the compounds described in this paper was reported previously in the literature, except for compound **3d** which has been recently prepared through a different procedure [17].

3. Biological results

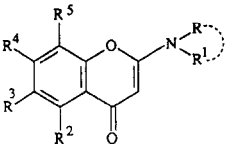
Compounds **1a,b**, **2a**, **3a–d**, **4a–c** and **13**, described in the present paper, were submitted to biological evaluation along with compounds **1c** [2], **2b,c** [21], **2d** [4], **2e** [22], **3e** [5], **4d** [6] and **10–12** [12], previously described by us. Also 2-morpholinochromone **1d**, whose antiproliferative properties were recently reported in the literature [15], has been examined as a reference compound.

All these compounds were tested in vitro for their activities using three different biological tests, i.e. the inactivation of T2 bacteriophage, the inhibition of DNA synthesis in Ehrlich ascites tumor cells and of the clonal growth capacity of HeLa cells cultivated in vitro.

Table 1
Structures of compounds **10–13**, **1a,b**, **2a**, **3a–c**, **4a,b**



10-13



1-4

Compound	R^2	R^3	R^4	R^5	$N^{R}_{R^1}$
10	H	H	H	H	-
11	-(CH=CH)$_2$-		H	H	-
12	H	H	-(CH=CH)$_2$-		-
13	H	-(CH=CH)$_2$-		CH_3	-
1a	H	H	H	H	NH-cyclopropyl
1b	H	H	H	H	NH-phenyl
2a	-(CH=CH)$_2$-		H	H	NH-cyclopropyl
3a	H	H	-(CH=CH)$_2$-		NH-cyclopropyl
3b	H	H	-(CH=CH)$_2$-		NH-phenyl
3c	H	H	-(CH=CH)$_2$-		$NHCH_3$
4a	H	-(CH=CH)$_2$-		CH_3	NH-cyclopropyl
4b	H	-(CH=CH)$_2$-		CH_3	NH-phenyl

3.1. T2 phage inactivation

Phage particles were incubated in the dark in the presence of increasing concentrations of the compounds tested and then the number of plaque forming units per ml was scored. This determination requires a large dilution of the virus suspension so that the indicator bacteria come into contact with a very reduced compound concentration. Thus, we can assume that there was no indirect effect on phage growth mediated by inhibition of some bacterial functions. We checked the consequences on bacterial growth induced by the compounds studied as described in the Section 5.2.2: all compounds were completely unable to affect the bacterial growth (data not shown).

Fig. 2 shows the results obtained on T2 bacteriophage. Compound **1d**, used as a reference, did not affect the infectivity of T2 bacteriophage. Compounds **2b,c**, **3b,e**, **4d** and **10–12** showed a similar behaviour, even if their activities were a little higher. Only compounds **2d** (Fig. 2(A)) and **4b** (Fig. 2(B)) were able to reduce the surviving fraction significantly. In particular, **4b** killed about 94% of the virions at a 16.59 μM concentration (5 μg/ml). The data related to the entirely inactive compounds are not presented in the figure.

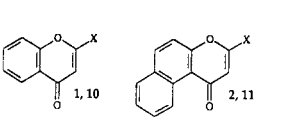
3.2. Effect on mammalian cells

The activity of the chromone derivatives was assayed in mammalian cells using two different tests. The first one is based on the determination of DNA synthesis in Ehrlich ascites tumor cells, a well-known transplantable tumor of mice; the second consists of a clonogenic assay performed with HeLa cells cultivated in vitro. The data of compounds **1a–c**, **2a–e**, **3a–e**, **4a–d** and **10–13**, and of reference compound **1d** are reported in Table 2.

3.3. Inhibition of DNA synthesis

The most effective compounds appeared to be **4a** and **2b**, followed by **11**, **10** and **2e**. A significant, even if less conspicuous, inhibition of DNA synthesis was also observed with

Table 2
Biological data of aminoderivatives **1a–d**, **2a–e**, **3a–e**, **4a–d** and (methylthio) derivatives **10–13**



Compound	X	Reference	Inhibition of DNA synthesis [a]	Inhibition of clonal growth [b]
1a	NH-cyclopropyl	#	nd	37.59±2.26
1b	NH-phenyl	#	nd	30.12±2.18
1c	$N(CH_3)_2$	[2]	nd	73.53±11.89
2a	NH-cyclopropyl	#	13.40±8.71	26.88±4.62
2b	NH-phenyl	[21]	1.98±0.70	39.06±5.49
2c	$NHCH_3$	[21]	54.64±1.79	32.68±14.95
2d	$N(CH_3)_2$	[4]	200.0±8.0	16.83±1.30
2e	N-morpholino	[22]	5.12±0.64	33.56±21.17
3a	NH-cyclopropyl	#	9.39±0.81	20.32±2.31
3b	NH-phenyl	#	8.21±0.66	39.68±10.08
3c	$NHCH_3$	#	333.33±0.03	nd
3d	N-morpholino	#	13.62±0.82	22.52±4.87
3e	$N(CH_3)_2$	[5]	74.63±5.57	nd
4a	NH-cyclopropyl	#	1.74±0.20	9.92±1.50
4b	NH-phenyl	#	7.37±0.93	43.48±8.70
4c	N-morpholino	#	nd	nd
4d	$N(CH_3)_2$	[6]	192.31±7.40	29.94±5.92
10	SCH_3	[12]	5.10±1.14	86.20±28.23
11	SCH_3	[12]	4.99±0.74	24.51±1.68
12	SCH_3	[12]	21.93±1.39	22.42±4.12
13	SCH_3	#	9.71±2.50	20.32±0.33
1d [c]	N-morpholino	[15]	19.16±17.29	45.04±8.11

[a] Expressed as IC_{50}, where IC_{50} is the drug concentration (μM) which induces a 50% inhibition of DNA synthesis in Ehrlich ascites tumor cells.
[b] Expressed as IC_{50}, where IC_{50} is the drug concentration (μM) which induces a 50% inhibition of the clonal growth in HeLa cells.
[c] Tested as a reference compound.
Described in the present paper.
nd: values not detectable.

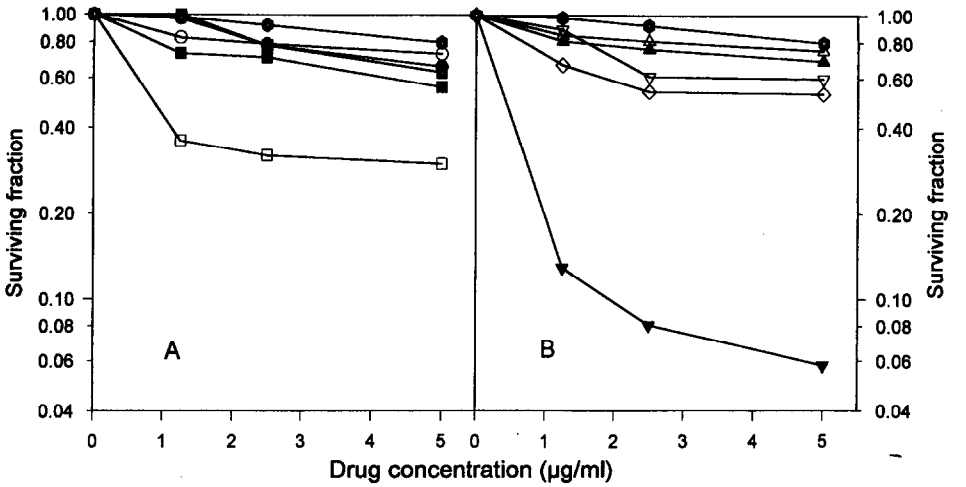


Fig. 2. T2 phage inactivation. Virus particles were incubated in the presence of increasing concentrations of chromone derivatives and then the number of the plaque forming units per ml was scored. (A) ○, **2b**; ●, **2c**, □, **2d**; ■, **10**; white cross in black square, **11**; white cross in black hexagon, **1d**. (B) △, **3b**; ▲, **3e**; ▼, **4b**; black dot in white lozenge, **4d**; ▽, **12**; white cross in black hexagon, **1d**.

3a, **3b**, **4b** and **13**. All these compounds were more active than the reference compound **1d**; an activity comparable with that of **1d** was shown by **2a**, **3d** and **12**. The remaining chromone derivatives induced insignificant or undetectable values of inhibition of DNA synthesis.

3.4. Inhibition of clonal growth

The picture achieved with this test is quite different from that obtained studying DNA synthesis; in fact, only compound **12** showed a similar activity in both tests. In general, we can see that the compounds tested appeared to be much less effective in the clonogenic assay than on DNA synthesis. Also the reference compound **1d** exhibited a reduced activity in this test. These data suggest that such chromone derivatives can induce a marked antiproliferative effect which, for many of them, is much higher than that provoked by **1d** and is not accompanied by significant cytotoxic effects.

However, it must be noted that the linear benzo-fused chromone derivative **4a** showed both a high activity as a DNA synthesis inhibitor and an appreciable cytotoxic effect on HeLa cells.

3.5. Inhibition of DNA, RNA and protein synthesis by compound 4a

Because compound **4a** appeared to be the most effective on DNA synthesis in Ehrlich cells, its antiproliferative effect was investigated further. Its capacity to inhibit RNA and protein synthesis was also determined. The data thus obtained are shown in Fig. 3. It is evident that compound **4a** can inhibit selectively DNA and RNA synthesis to a very similar extent, but is much less effective on protein synthesis.

4. Discussion

The bicyclic or tricyclic chromone derivatives **1a–c**, **2a–e**, **3a–e**, **4a–d** and **10–13** were studied using three different biological tests concerning the infectivity of T2 bacteriophage, the macromolecular synthesis in Ehrlich cells and the clonal growth capacity of HeLa cells.

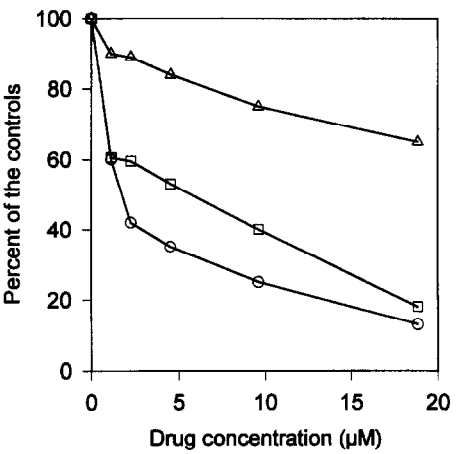


Fig. 3. Inhibition of macromolecular synthesis in Ehrlich cells by incubation in the presence of increasing concentrations of compound **4a**. The symbols are related to the synthesis of: ○, DNA; □, RNA; △, proteins.

In the first test the compound under examination was kept in contact only with the virions. In fact, to test virus infectivity, the viral suspensions were deeply diluted, so that the indicator bacteria were in contact with very low compound concentrations. Therefore, in this test we can observe only the direct effect of the drug on viral structures, e.g. on viral DNA before it is injected into the host bacteria. The two tests on mammalian cells give us information about the consequences of a short- or of a long-term treatment; actually, DNA, RNA or protein synthesis was assayed after one hour and a half, while in the clonogenic test the treatment was as long as 7 days. In other words, the assay on DNA synthesis gives information about the immediate antiproliferative effect, while the clonogenic test shows up the long-term cytotoxic effects.

In the T2 test the majority of the compounds were practically ineffective; only two of them were capable of reducing

the surviving fraction significantly, i.e. compounds **2d** and, principally, **4b**. We observe that they can also induce a certain cytotoxic effect on HeLa cells. To be understood, such a result requires further investigation because at present we have not sufficient information about it.

On the contrary, in mammalian cells the tested chromone derivatives generally proved to be effective antiproliferative drugs, even if to very different extents. The kind of substituent linked to the chromone nucleus plays an important role in this activity: for example, the methylthio, phenylamino and cyclopropylamino groups gave, on the whole, the best results, whereas the activity appeared to be lower with the methylamino and dimethylamino groups. However, a particular substituent can confer a pronounced or low activity to the compound according to the structure of the nucleus. For example, we observe that the presence of the cyclopropylamino substituent in a linear tricyclic nucleus (compound **4a**) confers a strong inhibitory activity on DNA synthesis, which is clearly reduced if this group is inserted in an angular one (compounds **2a** and **3a**), and is undetectable with the corresponding bicyclic derivative **1a**. In conclusion, the effectiveness seems to depend both on the type of X substituent (see Table 2) and on the structure of the cyclic system of the chromone derivative. We can say, roughly, that the bicyclic compounds gave the worst results as DNA synthesis inhibitors.

Actually, as mentioned before, in the test on HeLa cells the drug acts on them for a very long time (7 days), so that its toxicity is emphasized. In such severe experimental conditions only compound **4a** exhibited an IC_{50} value (9.92 μM) consistent with a significant cytotoxic effect, causing a 95.8% inhibition of the clonal growth at an 18.85 μM concentration (5 μg/ml). Considering that in the test on DNA synthesis several chromone derivatives exhibited an IC_{50} lower than 10 μM (and two lower than 2 μM), we can say that they induce an antiproliferative effect which is usually accompanied by a reduced cytotoxicity.

Finally, from the biological results obtained for the tested bicyclic and tricyclic chromone derivatives the following conclusions can be drawn.

1. Such compounds often possess, to a variable extent depending on the nucleus structure and the X substituent, significant antiproliferative properties but, except for **4a**, low cytotoxic activity.
2. Considering all the performed tests, the tricyclic structures **2** (angular) and **4** (linear) appear to be the most interesting ones.
3. Compound **4a** is the most active upon the whole, even if **2b** shows nearly equivalent antiproliferative properties and is free from significant cytotoxic activity.

5. Experimental

5.1. Chemistry

Melting points were determined using a Fisher-Johns apparatus (Electrothermal when above 300°C) and are uncorrected. IR spectra were recorded on a Perkin-Elmer 398 spectrophotometer (for the abbreviations relative to the IR bands see Table 3). ^{1}H NMR spectra were recorded on a Hitachi Perkin-Elmer R 600 spectrometer (60 MHz), and chemical shifts (δ) are reported in ppm using $(CH_3)_4Si$ as an internal reference ($\delta=0$). Analyses of all new compounds, indicated by the symbols of the elements, were within ±0.4% of the theoretical values and were performed by Laboratorio di Microanalisi, Dipartimento di Scienze Farmaceutiche, Università di Genova (University of Genoa, Italy).

Thin-layer chromatograms were run on Merck silica gel 60 F_{254} precoated plastic sheets (layer thickness 0.2 mm). Column chromatography was performed using Carlo Erba silica gel (0.05–0.20 mm) or Carlo Erba neutral aluminium oxide (Brockmann activity I).

5.1.1. Synthesis of naphthopyranones 3d, 6, 7 and 4c, 8, 9

Phosphorus oxychloride (75.0 mmol, 11.50 g) was added dropwise with stirring to an ice-cooled solution of 55.0 mmol (11.06 g) of ethyl 3-morpholino-3-oxopropanoate [22] in 10 ml of chlorobenzene. The resulting solution was stirred at room temperature for 30 min, and then a suspension of 50.0 mmol of the appropriate naphthol in 50 ml of chlorobenzene was added and the mixture was heated at 130°C for 4 h (compounds **3d**, **6** and **7**) or at 110°C for 10 h (compounds **4c**, **8** and **9**), while stirring. After cooling, a solution of 68 g of trihydrate sodium acetate in 150 ml of water was added and the resulting mixture was stirred at 60°C for 1 h. The organic layer was then collected and the aqueous phase was exhaustively extracted with chloroform. The combined organic phases were dried (anhydrous sodium sulfate) and the solvents removed in vacuo to give a thick oily residue from which compounds **3d**, **6**, **7** or **4c**, **8**, **9** were recovered through the procedures described below.

2-Morpholino-4H-naphtho[1,2-b]pyran-4-one ***3d***, *4-chloro-2H-naphtho[1,2-b]pyran-2-one* ***6*** *and 4-morpholino-2H-naphtho[1,2-b]pyran-2-one* ***7***. The residue derived from the reaction carried out with 7.22 g of 1-naphthol was treated with a little acetone to afford a first amount (3.54 g) of pure **3d** as a whitish solid; m.p. 265.5–266.5°C after crystallization from dichloromethane (Ref. [17]: m.p. 268–269°C). *Anal.* ($C_{17}H_{15}NO_3$) C, H, N. IR (KBr): 1640 (CO), 1624, 1605 br, 1560 cm^{-1}. ^{1}H NMR (CF_3COOD): δ 4.31 (near s, 8H, CH_2), 6.67 (s, 1H, H-3), 7.75–8.33 (m, 5H, H-5,6,7,8,9), 8.48 (m, 1H, H-10).

The filtrate was then evaporated to dryness under reduced pressure to give an oil which was chromatographed on a silica gel column. The first fractions eluted with a mixture of chloroform and ethyl acetate (1:1), after removal of solvents in vacuo, afforded an oil from which, after treatment with a little isopropyl ether, compound **6** (1.42 g, 12%) was obtained and identified by comparison (m.p., IR) with an authentic sample [12].

The fractions subsequently eluted with ethyl acetate, after removal of solvent, gave 1.27 g (9%) of nearly pure com-

pound **7**; pale-yellow crystals, m.p. 205–206°C after crystallization from ethyl acetate. *Anal.* ($C_{17}H_{15}NO_3$) C, H, N. IR (KBr): 1710 s, br (CO), 1628, 1595, 1550 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.31 (m, 4H, NCH_2), 3.96 (m, 4H, OCH_2), 5.82 (s, 1H, H-3), 7.42–8.06 (m, 5H, H-5,6,7,8,9), 8.60 (m, 1H, H-10).

By further elution with a mixture of chloroform and methanol (9:1), an additional crop (0.40 g) of pure **3d** was obtained (total yield 28%).

10-Methyl-2-morpholino-4H-naphtho[2,3-b]pyran-4-one ***4c****, 4-chloro-10-methyl-2H-naphtho[2,3-b]pyran-2-one* ***8*** *and 10-methyl-4-morpholino-2H-naphtho[2,3-b]pyran-2-one* ***9***. The thick oil obtained from the reaction carried out with 7.91 g of 1-methyl-2-naphthol [23] was dissolved in a little chloroform and chromatographed on a silica gel column eluting first with the mixture chloroform/ethyl acetate (1:1). The eluate collected was evaporated to dryness in vacuo to give a solid residue which was taken up in a little isopropyl ether and filtered; the compound so obtained, **8** (1.45 g, 12%), was identified by comparison (m.p., IR) with an authentic sample [6].

By eluting with ethyl acetate, nearly pure compound **9** (0.74 g, 5%) was then recovered; ivory-white crystals, m.p. 235°C, after crystallization from ethyl acetate. *Anal.* ($C_{18}H_{17}NO_3$) C, H, N. IR (KBr): 1692 s, br (CO), 1625, 1606, 1590 sh, 1556 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.77 (s, 3H, CH_3), 3.36 (m, 4H, NCH_2), 4.00 (m, 4H, OCH_2), 5.85 (s, 1H, H-3), 7.44–8.30 (m, 5H, H-5,6,7,8,9).

Further elution of the column with methanol finally afforded a dark, thick oil which was stirred at room temperature with a mixture of a little ethyl acetate and aqueous 6N HCl so that the solid hydrochloride of **4c** separated out. This was collected by filtration and suspended in aqueous 10% sodium carbonate, then the mixture was extracted several times with chloroform. From the combined extracts, after drying and removing the solvent, nearly pure compound **4c** (0.63 g, 4.3%) was obtained; whitish crystals, m.p. 239–240°C, after crystallization from acetone. *Anal.* ($C_{18}H_{17}NO_3$) C, H, N. IR (KBr): 1638 w, 1614 (CO), 1600 s, br, 1562 s, 1503 w cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.68 (s, 3H, CH_3), 3.61 (m, 4H, NCH_2), 3.88 (m, 4H, OCH_2), 5.50 (s, 1H, H-3), 7.59 (m, 2H, H-7,8), 8.05 (m, 2H, H-6,9), 8.64 (s, 1H, H-5).

5.1.2. 4-Methoxy-10-methyl-2H-naphtho[2,3-b]pyran-2-one ***14***

2.0 mmol (0.49 g) of compound **8** were dissolved in 150 ml of boiling dry methanol. After cooling, the solution was added to a methanolic solution of sodium methoxide (4.0 mmol (0.09 g) of sodium in 50 ml of dry methanol). The mixture was stirred at room temperature for 30 min, then poured into 400 ml of water previously acidified with diluted aqueous hydrochloric acid. The white solid compound **14** that separated out was collected by filtration, washed with water and dried (0.46 g, 96%); white crystals, m.p. 187–188°C, after crystallization from ethyl acetate/isopropyl ether (1:1).

Anal. ($C_{15}H_{12}O_3$) C, H. IR (KBr): 1718 s, br (CO), 1626, 1618, 1598 sh, 1564 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.67 (s, 3H,10-CH_3), 4.02 (s, 3H, OCH_3), 5.70 (s, 1H, H-3), 7.24–8.37 (m, 5H, H-5,6,7,8,9).

5.1.3. 4-Methoxy-10-methyl-2H-naphtho[2,3-b]pyran-2-thione ***15***

A mixture of 2.0 mmol (0.48 g) of compound **14**, 3.0 mmol (1.21 g) of Lawesson's reagent and 40 ml of anhydrous toluene was heated at 120°C for 3 h, while stirring. The solvent was then removed under reduced pressure and the residue was taken up in a little dichloromethane and chromatographed on a neutral aluminium oxide column, eluting with the same solvent. The eluate collected was evaporated to dryness in vacuo to give 0.48 g (94%) of pure compound **15** as a yellow solid; m.p. 222–223°C after crystallization from ethyl acetate.

Anal. ($C_{15}H_{12}O_2S$) C, H, S. IR (KBr): 1620 sh, 1606, 1588, 1548, 1104 (CS) cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.82 (s, 3H, 10-CH_3), 4.08 (s, 3H, OCH_3), 6.83 (s, 1H, H-3), 7.46–8.39 (m, 5H, H-5,6,7,8,9).

5.1.4. 4-Hydroxy-10-methyl-2H-naphtho[2,3-b]pyran-2-thione ***16***

A mixture of 3.0 mmol (0.77 g) of compound **15** and 15 g of pyridine hydrochloride was heated at 230°C, under nitrogen. After melting of the mixture, the solution obtained was further stirred at 230°C for 5 min, then allowed to cool. The resulting solid mixture was treated with water (30 ml) at room temperature until the nearly pure compound **16** separated out as amorphous solid which was recovered by filtration, washed with water and dried (0.70 g, 96%); dark-yellow crystals, m.p. > 360°C, after crystallization from methanol.

Anal. ($C_{14}H_{10}O_2S$) C, H, S. IR (KBr): 3100–2400 br, s, complex (OH), 1624, 1608, 1583, 1533 br, 1102 s (CS) cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.78 (s, 3H, CH_3), 6.73 (s, 1H, H-3; partially disappeared with D_2O), 7.56–7.83 (m, 2H, H-7,8), 8.18 (m, 2H, H-6,9), 8.46 (s, 1H, H-5).

5.1.5. 10-Methyl-2-(methylthio)-4H-naphtho[2,3-b]pyran-4-one ***13***

A mixture of 5.0 mmol (1.21 g) of compound **16**, 0.70 g of anhydrous potassium carbonate, 1.0 ml of methyl iodide, and 40 ml of dry acetone was refluxed for 1 h. The solvent was then removed in vacuo and the residue partitioned between water and warm chloroform. The aqueous phase was extracted several more times with chloroform. The combined organic extracts were dried (anhydrous sodium sulfate), then evaporated to dryness under reduced pressure to give a nearly solid residue which was treated with a little ethyl acetate so that pure compound **13** separated out (0.95 g, 74%); yellow crystals, m.p. 227–228°C, after crystallization from dichloromethane/ethyl acetate.

Anal. ($C_{15}H_{12}O_2S$) C, H, S. IR (KBr): 1638, 1616 s (CO), 1600, 1550, 1500 w cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.63 (s, 3H, SCH_3), 2.79 (s, 3H, 10-CH_3), 6.26 (s, 1H, H-3), 7.69 (m, 2H, H-7,8), 8.11 (m, 2H, H-6,9), 8.70 (s, 1H, H-5).

*5.1.6. N-Monosubstituted 2-amino-4H-1-benzopyran-4-ones **1a,b**, 3-amino-1H-naphtho[2,1-b]pyran-1-one **2a**, 2-amino-4H-naphtho[1,2-b]pyran-4-ones **3a–c** and 2-amino-10-methyl-4H-naphtho[2,3-b]pyran-4-ones **4a,b***

*N-Cyclopropyl substituted compounds **1a**, **2a**, **3a**, **4a**.* A mixture of 2.0 mmol of (methylthio) derivative **10** [12] (0.38 g), **11** [12] (0.48 g), **12** [12] (0.48 g) or **13** (0.51 g), 20.0 mmol (1.14 g) of cyclopropylamine and 10 ml of ethylene glycol was heated at 160°C for 1 h, with stirring. The resulting solution was poured into ice-water and the mixture was exhaustively extracted with chloroform. The combined extracts (dried over anhydrous sodium sulfate) were evaporated to dryness in vacuo to give a thick oily residue from which, after treatment with a little ethyl ether, the respective nearly pure compounds **2a**, **3a**, or **4a**, separated out as whitish solids. Only in the case of **1a**, the oily residue was chromatographed on a silica gel column, eluting with ethyl acetate, and the fraction collected was evaporated to dryness under reduced pressure to give the pure compound as a white solid.

By crystallizing these compounds from the suitable solvents, white or whitish crystals were obtained.

*N-Methyl substituted compound **3c**.* A mixture of 2.0 mmol (0.48 g) of compound **12** [12] , 40.0 mmol (2.70 g) of methylamine hydrochloride, 40.0 mmol (4.05 g) of triethylamine and 50 ml of ethanol was refluxed for 16 h, with stirring. The mixture was then evaporated to dryness in vacuo and the resulting solid residue was partitioned between 10% aqueous sodium carbonate and dichloromethane. The organic layer was collected and the aqueous phase was further extracted twice with dichloromethane. The combined extracts (dried over anhydrous sodium sulfate), after removal of solvent, afforded a solid residue which was taken up in a little ethyl ether to yield pure compound **3c**, a white solid which was crystallized from dichloromethane/ethyl acetate.

*N-Phenyl substituted compounds **1b**, **3b**, **4b**.* A mixture of 2.0 mmol of (methylthio) derivative **10** [12] (0.38 g), **12** [12] (0.48 g) or **13** (0.51 g), 40.0 mmol (3.73 g) of aniline, 0.15 g of monohydrate *p*-toluenesulfonic acid and 10 ml of ethylene glycol was heated at 160°C for 1h, with stirring. The resulting solution was then poured into ice-water (200 ml), so that a dark oil separated out. After adding 50 ml of ethyl ether/petroleum ether 40–70°C (1:1) and vigorously stirring the mixture at room temperature for 30 min, a solid separated which was collected by filtration, washed with water and a little ethyl ether, then dried. The respective nearly pure compounds **1b**, **3b** or **4b** so obtained were then crystallized from the suitable solvents to give white (**1b**, **3b**) or pale-yellow (**4b**) crystals.

Data for compounds **1a,b**, **2a**, **3a–c** and **4a,b** are reported in Table 3.

5.2. Biology

5.2.1. Chemicals

Test compounds were dissolved in DMSO (4.5 mmol/l) and the solutions were stored frozen in plastic tubes. Just before the experiments were performed, a calculated amount of the compound solution was added to PBS or to the growth medium containing cells to a final solvent concentration of 0.5%. [^{3}H]-thymidine (4.77 TBq/mmol), [^{3}H]-uridine (1.1 TBq/mmol) and [^{3}H]-leucine (2.37 TBq/mmol) were obtained from Amersham International (UK).

5.2.2. T2 bacteriophage and Escherichia coli B48 inactivation

T2 phage was grown in *E. coli* B48 cultures using nutrient broth. Virus suspensions (10^8–10^9 particles per ml) in 1 mM $MgSO_4$ containing the compound to be tested were incubated at 37°C for 60 min. After appropriate dilutions (generally from 10^{-5} to 10^{-7}), virus titres were determined according to Adams [24], using *E. coli* B48 as indicator bacteria and brain–heart infusion agar.

The effect of the compounds tested on bacteria growth was determined by incubating aliquots of 10^4/ml *E. coli* B48 cells in their presence for 2 h and then checking cell density in comparison with the untreated control and a positive one treated with chloramphenicol (2.5 μg/ml). Bacterial cell density was determined by light scattering at 440 nm.

5.2.3. Macromolecular synthesis in Ehrlich cells

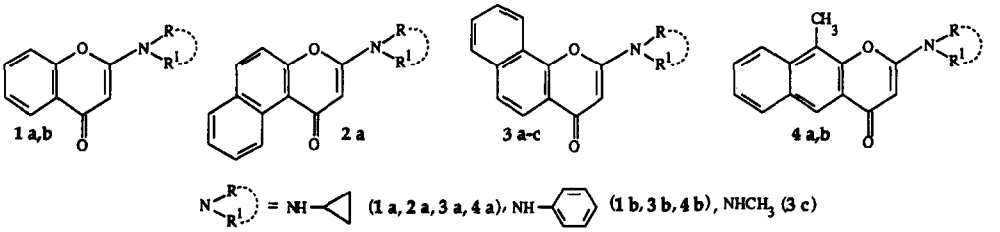
Ehrlich ascites tumor cells (Lettrè strain from Heidelberg) were routinely transferred by injecting intraperitoneally 2×10^6 cells per animal into NCL mice. The tumor cells, collected on days 6–7 after transplant, were processed as already described in Ref. [25].

2×10^7 cells/ml in Hank's solution containing the compound to be studied were incubated at 37°C for 60 min; then 40 kBq/ml of [^{3}H]-thymidine, [^{3}H]-uridine or [^{3}H]-leucine (for DNA, RNA or protein synthesis, respectively), in a small volume of the same medium, were added and the cells were further incubated at 37°C for 30 min. The acid-insoluble fraction was then precipitated by adding 5% ice-cold trichloroacetic acid and filtered through Whatman GF/C filters. After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. The results were calculated as the percentage of radioactivity incorporated into untreated control cells (about 3–6 MBq); filtrations were carried out on a Sample Manifold apparatus (Millipore, Bedford, MA, USA).

5.2.4. HeLa cell culture

HeLa cells (kindly provided by Professor F. Majone, Department of Biology, Padua University, Italy) were grown as monolayers in nutrient mixture F12 Ham medium (Sigma)

Table 3
Data of *N*-monosubstituted 2-amino-4*H*-1-benzopyran-4-ones **1a,b**, 3-amino-1*H*-naphtho[2,1]-bipyran-1-one **2a**, 2-amino-4*H*-naphtho[1,2-b]pyran-4-ones **3a–c** and 2-amino-10-methyl-4*H*-naphtho[2,3-b]pyran-4-ones **4a,b**



Comp.	Yield (%)	M.p. (°C) (solvent) [a]	Molecular formula [b]	IR [c] (cm^{-1})	^1H NMR [d]: δ (ppm)
1a	81	150–150.5 (A)	$C_{12}H_{11}NO_2$	3185 br (NH), 1610 br, s (CO), 1552 br, 1520	0.59–1.01 (m, 4H, CH_2), 2.53 (m, 1H, N-CH), 5.76 (s, 1H, H-3), 6.90–7.73 (m, 4H, H-6,7,8 + NH; 3H after treatment with D_2O), 8.19 (m, 1H, H-5)
1b	58	217–218 (B)	$C_{15}H_{11}NO_2$	3150 br (NH), 1610 s (CO), 1598 sh, 1545 sh, 1520 s, br	5.59 (s, 1H, H-3), 7.00–7.80 (m, 8H, H-6,7,8 + phenyl Hs), 8.05 (m, 1H, H-5), 10.12 [e] (s, 1H, NH)
2a	52	227.5–228.5 (C)	$C_{16}H_{13}NO_2$	3420 (NH), 1632 (CO), 1603 w, 1590, 1567, 1500	0.63–1.10 (m, 4H, CH_2), 2.58 (m, 1H, N–CH), 5.83 (s, 1H, H-2), 6.39 [e] (near s, 1H, NH), 7.25–8.20 (m, 5H, H-5,6,7,8,9), 10.30 (m, 1H, H-10)
3a	64	196–197 (B)	$C_{16}H_{13}NO_2$	3230 br (NH), 1638 (CO), 1605 s, br, 1553, 1505	0.70–1.10 (m, 4H, CH_2), 2.67 (m, 1H, N–CH), 5.72 [e] (near s, 1H, NH), 5.90 [f] (s, 1H, H-3), 7.50–8.10 (m, 4H, H-6,7,8,9), 8.12–8.55 (m, 2H, H-5,10)
3b	59	264–265 (C)	$C_{19}H_{13}NO_2$	3200 br (NH), 1641 (CO), 1608 sh, 1592, 1550 br, 1496	5.73 [f] (s, 1H, H-3), 7.12–8.26 (m, 10H, H-5,6,7,8,9 + phenyl Hs), 8.42 (m, 1H, H-10), 10.21 [e] (s, 1H, NH)
3c	61	236–237 (D)	$C_{14}H_{11}NO_2$	3260 (NH), 1641 (CO), 1607 s, br, 1558, 1507	2.96 (s, 3H, CH_3), 5.40 (s, 1H, H-3), 7.67–8.35 (m, 6H, H-5,6,7,8,9 + NH; 5H after treatment with D_2O), 8.50 (m, 1H, H-10)
4a	55	257–258 (D)	$C_{17}H_{15}NO_2$	3420 (NH), 1607 (CO), 1566, 1506	0.67–1.03 (m, 4H, CH_2), 2.50–2.90 (m, 1H, N-CH), 2.76 (s, 3H, CH_3), 5.67 (s, 1H, H-3), 7.43–7.85 (m, 3H, H-7,8 + NH; 2H after treatment with D_2O), 8.07 (m, 2H, H-6,9), 8.58 (s, 1H, H-5)
4b	79	287–288 (C)	$C_{20}H_{15}NO_2$	3155 br (NH), 1627 w, 1611 s (CO), 1590 s, 1562 s	2.72 (s, 3H, CH_3), 5.57 (s, 1H, H-3), 7.23–7.82 (m, 7H, H-7,8 + phenyl Hs), 8.18 (m, 2H, H-6,9), 8.53 (s, 1H, H-5), 10.22 [e] (s, 1H, NH)

[a] Crystallization solvent: A = ethyl acetate/petroleum ether 40–70°C; B = ethyl acetate; C = ethanol; D = dichloromethane/ethyl acetate.
[b] *Anal.* C, H, N.
[c] In KBr pellets, except for **2a** and **4a** ($CHCl_3$). Abbrevations: br = broad, s = strong, sh = shoulder, w = weak.
[d] Solvents: $CDCl_3$ for **1a**, **2a**, **3a**; DMSO-d_6 for **1b**, **3b**, **3c**, **4a**, **4b**.
[e] Disappeared with D_2O.
[f] Partially disappeared with D_2O.

supplemented with 10% foetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel) and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml) [26]. Trypsin (0.25%, Boehringer, Mannheim) was routinely used for subculture.

5.2.5. Clonogenic survival

Trypsinized HeLa cells were grown at a density of 200 cells in a 60 mm dish. Triplicate cultures were established for each treatment. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration; cells were incubated for 7 days at 37°C in a 5% carbon dioxide atmosphere. After this time the colonies were stained and counted, discarding colonies with less than 50 cells. The efficiency of the clonal growth, i.e. the ratio between the number of colonies formed and the number of cells seeded, was then calculated and used to normalize the cytotoxicity induced by the compounds. For untreated cells the cloning efficiency was about 90%.

5.2.6. Radiochemical determinations

The radioactivity measurements were performed using Instagel (Packard Instruments, Meriden, CT, USA) as a scintillation fluid. All determinations were carried out with a Packard A 300 CD spectrometer. Double-isotope counting was accomplished automatically on the bases of quenching curves obtained using [^3H]-radioactivity standards.

5.2.7. Calculations

The data related to DNA synthesis were elaborated using probit analysis, thus obtaining the IC_{50}, i.e. the compound concentration (μmol/l) which induces a 50% inhibition of DNA synthesis. All the biological experiments were carried out at least in triplicate.

Acknowledgements

The authors wish to thank Mr A. Panaro for elemental analyses, Dr C. Rossi for ^{1}H NMR spectra, and Mr F. Tuberoni for IR spectra. Financial support from Consiglio Nazionale delle Ricerche, Rome, Italy, is gratefully acknowledged.

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