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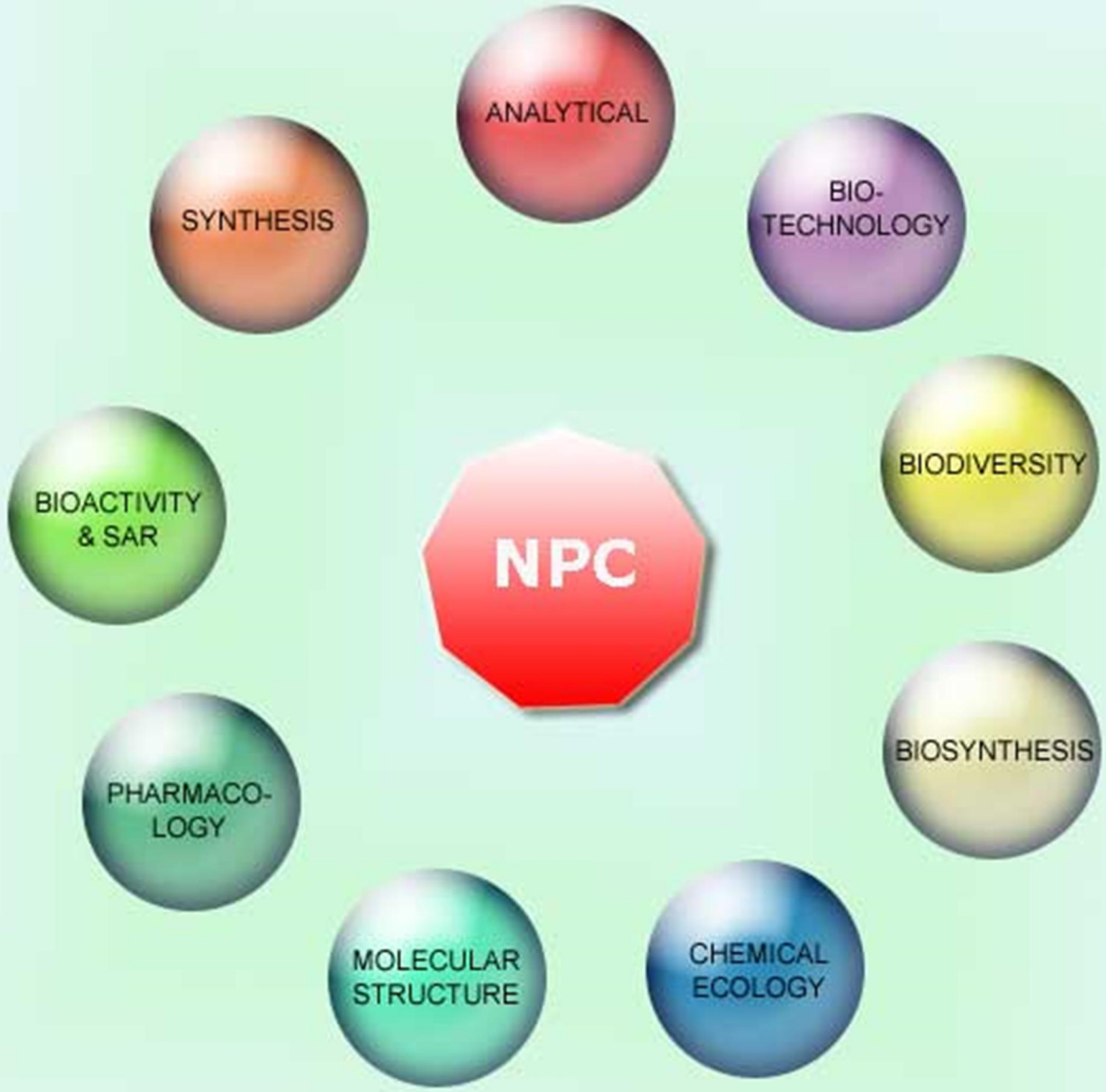

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Cytotoxic and Antitumor Activity of some Coumarin Derivatives

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Several natural and synthetic coumarins were assayed against different cancer cell lines. Four of them have shown cytotoxicity against a panel of three human solid tumor cell lines (HeLa, T-47D, and WiDr) and a clearly activity/hydrophobicity relationship. Compound **13** proved to be the most active product in all cell lines tested, with values of 8.0 (±0.38) μM against HeLa cells and also able to inhibit *Taq* DNA polymerase. This dual activity of **13** makes it a candidate to be considered as a "lead" compound in the search for novel antitumor drugs.

Keywords: Coumarins, Antitumor, Cell lines, Replication inhibition, Lipophilicity. Coumarins, Antitumor.

Cancer is still the second leading cause of death worldwide. Numerous experiments have been going on to develop compounds having minor or no side effects; and coumarins were reported to exhibit these properties [1].

Synthetic or natural coumarins are a versatile class of aromatic heterocycles and constitute a relevant class of pharmacological agents presenting a wide range of different functionalizations [2]. Their antitumor properties include the inhibition of the cell cycle, thus conferring chemoprevention of cancer [3, 4]. The antitumor activities of coumarins and its known metabolite, umbelliferone (7-hydroxycoumarin), were tested against three human carcinoma cell lines {colon-carcinoma cell line (Caco-2), a hepatoma-derived cell line (Hep-G2) and a lymphoblastic cell line (CCRF cem)} [5]. Coumarins, represented by 2,4-diaryl-4H,5H-pyrano[3,2-c]benzopyran-5-ones, have displayed strong anti-proliferative activities in MCF-7 breast carcinoma cells by a mechanism that remains to be determined [6].

The search for new leads can also be made by combinatorial chemistry or semi-synthesis reactions. Although it is possible with these techniques to produce quickly new collections of compounds of various size and composition which increase molecular diversity, the generation of new defined stereogenic centers is an arduous labor [7].

In this work we report the cytotoxic and antiproliferative activities of a small set of natural and synthetic coumarin derivatives. Some of them have shown DNA polymerase inhibition previously [8, 9]. In order to perform structure-activity relationship (SAR) studies against DNA replication and antitumor activity, we envisioned different points of modification on the molecular scaffold.

The *in vitro* antiproliferative activity of twenty compounds was evaluated using the National Cancer Institute (NCI) protocol, after 48 h of drug exposure using the sulforhodamine B (SRB) assay, including the commercial drugs coumarin (**18**), 4-hydroxy (**19**), and 6-hydroxycoumarin (**20**) [10-12]. The results expressed as 50% growth inhibition (GI_{50}) are shown in Table 1. From the GI_{50} values, the structure-activity relationships clearly show a direct relationship between lipophilicity and activity [13].

Compounds **1** and **2** were isolated from plants belonging to the *Pterocaulon* genus (Asteraceae) and **3** was obtained using NBS in CH_2Cl_2 (Figure 1). Product **3** was able to inhibit *Taq* DNA polymerase, but did not show antitumor activity. Its loss of action could be attributed to its remarkably polarity. This halohydrin has a particular structural feature in its side chain, showing polar groups (alcohol and halogen) able to form an intramolecular hydrogen bond with a partial five membered cycle.

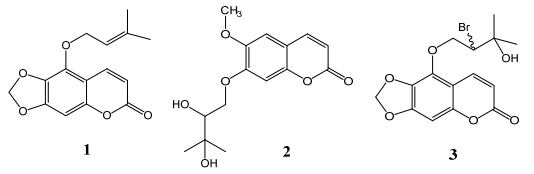

Figure 1: Natural coumarins from *Pterocaulon* sp. and halohydrin derivative assayed.

Using 4-hydroxycoumarin as starting material and either allyl or γ,γ-dimethylallyl bromine, we could obtain compounds **4**-**9**. The derivatives **4** and **9** were liquid oils, due to their low polarity. Thus, but not surprisingly, only these two bi-substituted products exhibited inhibition of growth and cell division. Once again, their potential permeability through membranes seems to be crucial for their activity.

Cytotoxic drugs continue to play a crucial role in cancer therapy. Certain cytotoxic agents have been improved via pro-drug approaches. Improvements have been decidedly specific for drugs and disease, but have drawbacks with respect to the accuracy of cellular uptake and drug release. Among the strategies proposed to enhance the passive internalization of drugs into cells, increasing their lipophilicity has often been validated as a successful approach [14].

Taking this into account, we envisaged the preparation of more hydrophobic derivatives using 6-hydroxycoumarin as starting material. Compounds **10** and **11** were obtained via silicon protecting groups, and **12** in a classical acetylation reaction. The less polar of these products (**10**) showed *in vitro* antiproliferative activity against representative human solid tumor cell lines. Thus, functionalized chemical groups containing silicon atoms might just provide lipophilicity to the drug, permitting it to pass through the cell membrane by passive diffusion. This strategy has been successfully implemented in the antitumor drug analogs silaplatins (cisplatin analogs) [15,16] and silatecans (silicon-containing camptothecins) [17].

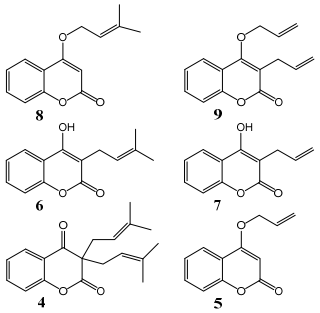

Figure 2: Synthesized allyl coumarins assayed.

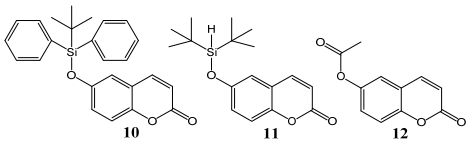

Figure 3: 6-Hydroxycoumarin derivatives evaluated.

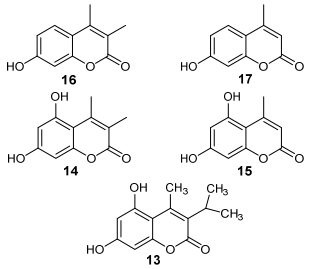

Figure 4: Assayed coumarins synthesized using Pechmann reaction

In order to increase the number of compounds for biological tests we prepared different semi-synthetic coumarins using the von Pechmann reaction variant [8] in the presence of ionic liquids. Products **13**-**17** show some degree of polarity, attributable to the presence of phenolic moieties. Compound **13** (the most hydrophobic of these five products) was active against all the tested cell lines and against DNA replication.

This dual activity of **13** makes this coumarin a good candidate to be considered as a "lead" product in the search of novel antitumor drugs, and its target of action could be nuclear DNA polymerases.

Coumarins target a number of pathways in the cancer field, such as kinase inhibition, cell cycle arrest, angiogenesis inhibition, heat shock protein (HSP90) inhibition, telomerase inhibition, antimitotic activity, carbonic anhydrase inhibition, monocarboxylate transporters inhibition, aromatase inhibition and sulfatase inhibition [1]. DNA polymerase inhibition affects the unlimited replicative potential of cancer cells by inhibiting DNA replication, and consequently antitumor activity, in leukemic and colon cancer cell lines for instance. There is a close evolutionary relationship between DNA dependent enzymes (such as human DNA polymerases) and *Taq* DNA polymerase. These structures resemble a right hand domain and conserve the amino acid sequence inside the catalytic region [18, 19]. It is very likely that the majority of effects observed on cellular divisions can be mediated by the reaction of DNA related enzymes with biomolecules such as proteins, peptides, and, at higher concentrations, DNA or chemical drugs.

In summary, the effects over cell viability were tested *in vitro* on several tumor cell lines, as well as in previous work against *Taq* DNA polymerase. Four products from a family of twenty coumarins have shown cytotoxic and antitumor activity in μM concentrations. Furthermore, compound **13** was able to inhibit both DNA replication and cell division in the three cancer cell lines assayed.

Table 1: *In vitro* antiproliferative activity against representative human solid tumor cell lines.[a]

	Cell line			cLog*P*[b]
Compounds	HeLa	T47D	WiDr	
1	na	na	na	3.32
2	na	na	na	1.01
3	na	na	na	2.64
4	17.6 (±6.4)	26.0 (±5.1)	21.60 (±5.9)	5.15
5	na	na	na	2.22
6	na	na	na	3.21
7	na	na	na	2.28
8	na	na	na	3.27
9	17.5 (±1.5)	54.3 (±4.7)	21.6 (±7.6)	3.38
10	24.1 (±4.1)	26.6 (±2.3)	32.2 (±1.8)	5.49
11	na	na	na	4.19
12	na	na	na	1.48
13	8.0 (±3.9)	27.0 (±7.4)	22.6 (±3.6)	2.00
14	na	na	na	1.32
15	na	na	na	0.88
16	na	na	na	1.67
17	na	na	na	1.23
18	na	na	na	nc
19	na	na	na	nc
20	na	na	na	nc

[a] Values, expressed as GI_{50} (50% growth inhibition), are given in μM and are means of three to five experiments; standard deviation is given in parentheses.
[b] The detected cLog *P* values were implemented using ALOGPS 2.1 program that is publicly available.

Experimental

General procedures: All the chemicals used were of analytical grade. Reactions requiring anhydrous conditions were performed under nitrogen. Dichloromethane and diethyl ether were distilled from CaH_2 and Na/benzophenone, respectively, under N_2 prior to use. TLC was carried out on Merck aluminum sheets coated with silica gel 60 F_{254}. Plates were visualized by use of UV light and/or sodium permanganate 20% solution without heating. NMR spectra were measured at 500, 400 or 200 MHz (^{1}H NMR) and 100 or 50 MHz (^{13}C NMR) in either $CDCl_3$ or MeOD, and chemical shifts are reported relative to internal Me_4Si (d = 0).

Data for assayed coumarins are listed below and in the previously publications [8, 9].

5-(3',3'-Dimethylallyloxy)-6,7-methylendioxycoumarin (**1**)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.78-1.73 (6H, bs, Me), 4.83 (bs, 1H, H-1´a), 4.87 (1H, d, *J*= 7.2 Hz, H-1´b), 5.47 (1H, bs, H-2´), 6.01 (2H, s, O-CH_2-O), 6.19 (1H, d, *J*= 9.6 Hz, H-3), 6.52 (1H, s, H-8), 7.96 (1H, d, *J*= 9.6 Hz, H-4)
^{13}C NMR from HSQC (50.6 MHz, $CDCl_3$): δ 17.00 (CH_3), 29.30 (CH_3), 69.00 (CH_2), 92.52 (CH-8), 101.60 (CH_2), 111.88 (CH-3), 119.53 (CH_2), 139.30 (CH-4).

7-(3'-Methyl-2',3'-dihydroxybutoxy)-6-methoxycoumarin (**2**)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.34-1.26 (6H, bs, Me), 3.89 (1H, bs, H-2´), 3.92 (3H, s, OMe), 4.30-4.15 (2H, bs, H-1´), 6.25 (1H, d, *J*= 9.8 Hz, H-3), 6.85 (1H, s, H-5), 6.91 (1H, bs, H-8), 7.62 (1H, d, *J*= 9.8 Hz, H-4)
^{13}C NMR from HSQC: δ (50.6 MHz, $CDCl_3$) 26.00 (CH_3), 27.50 (CH_3), 68.40 (OMe), 70.60 (CH_2), 74.80 (CH-2´), 97.60 (CH-5), 103.40 (CH-8), 110.99 (CH-3), 142.30 (CH-4).

5-(2´-Bromine-3´-hydroxy-3'-methyl-butyloxy)-6,7-methylenedi-oxycoumarin (**3**)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.44-1.25 (6H, bs, Me), 3.34 (1H, bs, OH), 4.22 (1H, dd, *J*= 3.2; 5.6 Hz, H-2´), 4.50 (1H, dd, *J*= 8.8; 2.8 Hz, H-1´b), 5.02 (1H, dd, *J*= 3.2; 8.0 Hz H-1´a) 6.05 (2H, s, O-

CH_2-O), 6.22 (1H, d, *J*= 9.8 Hz, H-3), 6.56 (1H, s, H-8), 8.12 (1H, d, *J*= 9.8 Hz, H-8)
^{13}C NMR from HSQC: δ (50.6 MHz, $CDCl_3$) 30.00 (CH_3), 64.20 (CH-2′), 74.10 (CH_2), 93.22 (CH-8), 101.90 (CH_2), 111.92 (CH-3), 139.80 (CH-4).

3,3-Bis(3-methylbut-2-en-1-yl)chromane-2,4-dione (4)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.68-1.51 (12H, s, Me), 2.75 (4H, bs, H-1′), 4.90 (2H, bs, H-2′), 7.25 (1H, bs, H-6), 7.52 (1H, bs, H-8), 7.63 (1H, dd, *J*= 1.8; 7.2 Hz, H-7), 7.90 (1H, dd, *J*= 1.6; 7.6 Hz, H-5)
^{13}C NMR (50.6 MHz, $CDCl_3$): δ 25.93-18.07 (CH_3), 37.55 (CH_2), 62.46 (C), 117.5 (CH-8), 117.64 (CH-2′), 124.84 (CH-6), 126.84 (CH-5), 137.02 (CH-7), 137.23 (C), 155.04 (C), 170.69 (C=O), 194.72 (C=O).

4-Allyloxy-coumarin (5)
^{1}H NMR (200 MHz, $CDCl_3$): δ 4.70 (2H, dd, *J*= 1.2; 5.0 Hz, H-1′), 5.48 (2H, bs, H-3′), 5.69 (1H, s, H-3), 6.13 (1H, m, H-2′), 7.27 (1H, bs, H-6), 7.32 (1H, bs, H-8), 7.52 (1H, dd, *J*= 1.6; 7.2 Hz, H-7), 7.86 (1H, dd, *J*= 1.4; 7.8 Hz, H-5)
^{13}C NMR from HSQC (50.6 MHz, $CDCl_3$): δ 69.00 (CH_2), 91.00 (CH-3) 117.05 (CH-8), 119.00 (CH-3′), 123.00 (CH-5), 124.05 (CH-6), 131.00 (CH-2′), 134.00 (CH-7).

3-(3',3'-Dimethylallyl)-coumarin (6)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.80-1.71 (6H, s, Me), 2.65 (2H, *d*, *J*= 7.2 Hz, H-1′), 5.04 (1H, bs, H-2′), 7.36 (1H, bs, H-8), 7.41 (1H, bs, H-6), 7.64 (1H, bs, H-7), 7.86 (1H, dd, *J*= 1.4; 7.8 Hz, H-5).

4-Hydroxy-3-allyl-coumarin (7)
^{1}H NMR (200 MHz, $CDCl_3$): δ 3.50 (2H, d, *J*= 6.8 Hz, H-1′), 5.27 (2H, bs, H-3′), 6.03 (1H, bs, H-2′), 7.27 (2H, bs, H-6; H-8), 7.51 (1H, dd, *J*= 1.6; 7.2 Hz, H-7) 7.81 (1H, d, *J*= 8.0 Hz, H-5)
^{13}C NMR from HSQC (50.6 MHz, $CDCl_3$): δ 29.00 (CH_2), 116.15 (CH-8), 117.20 (CH-3′), 121.89 (CH-5), 123.05 (CH-6), 134.23 (CH-7), 135.76 (CH-2′).

4-(3',3'-Dimethylallyloxy)-coumarin (8)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.84-1.78 (6H, s, Me), 4.68 (2H, d, *J*= 7.4 Hz, H-1′), 5.51 (1H, bs, H-2′), 5.68 (1H, s, H-3), 7.25 (1H, bs, H-6), 7.33 (1H, bs, H-8), 7.54 (1H, dd, *J*= 1.8; 7.2 Hz, H-7), 7.83 (1H, dd, *J*= 1.6; 8.0 Hz, H-5).

4-Allyloxy-3-allyl-coumarin (9)
^{1}H NMR (200 MHz, $CDCl_3$): δ 3.40 (2H, d, *J*= 6.2 Hz, H-1′′), 4.63 (2H, *d*, *J*=5.6 Hz, H-1′), 5.07 (2H, bs, H-3′′) 5.37 (2H, bs, H-3′) 5.99 (1H, bs, H-2′′), 6.08 (1H, bs, H-2′), 7.30 (1H, bs, H-6), 7.36 (1H, bs, H-8), 7.50 (1H, dd, *J*= 1.6; 7.6 Hz, H-7), 7.70 (1H, dd, *J*= 1.6; 8.0 Hz, H-5).

6-(t-Butyldiphenylsilyloxy)-coumarin (10)
^{1}H NMR (200 MHz, $CDCl_3$) δ 1.11 (9H, s, *t*-Bu), 6.31 (1H d, *J*= 9.6 Hz, H-3), 6.79-7.72 (14H, aromatic).

6-(Di-t-butylsilyloxy)-coumarin (11)
^{1}H NMR (200 MHz, $CDCl_3$): δ 1.06 (18H, s, 2*t*-Bu), 4.45 (1H, s, H-Si), 6.41 (1H, d, *J*= 9.6 Hz, H-3), 6.98-7.22 (3H, bs, H5, 7, 8), 7.62 (1H, d, *J*= 9.6 Hz, H-4).

6-Acetoxycoumarin (12)
^{1}H NMR (200 MHz, $CDCl_3$): δ 2.33 (3H, s, Ac), 6.46 (1H, d, *J*= 9.6 Hz, H-3), 7.28 (3H, bs, H5, 7, 8), 7.66 (1H, d, *J*= 9.6 Hz, H-4).

3-Isopropyl-4-methyl-5,7-dihydroxycoumarin (13)
^{1}H NMR (400 MHz, MeOD): δ 1.3 (6H, d, *J*= 9.0 Hz, 2Me), 2.65 (3H, s, Me), 3.30 (1H, m, CH), 6.18 (1H, d, *J*= 2.0 Hz, H-8), 6.23 (1H, d, *J*= 2.0 Hz, H-6).
^{13}C NMR (100 MHz, MeOD): δ 17.52 (CH_3), 18.82 ($2CH_3$), 27.37 (CH-2′$_{i\text{-Pr}}$), 94.15 (CH-8), 99.16 (CH-6), 103.37 (C), 124.20 (C), 149.96 (C), 155.04 (C), 157.66 (C), 160.07 (C), 161.16 (C=O).

3,4-Dimethyl-5,7-dihydroxycoumarin (14)
^{1}H NMR (400 MHz, MeOD): δ 2.08 (3H, s, Me), 2.58 (3H, s, Me), 6.19 (1H, d, *J*= 2.0 Hz, H-8), 6.22 (1H, d, *J*= 2.0 Hz, H-6).
^{13}C NMR (100 MHz, MeOD): δ 11.24 (CH_3), 18.07 (CH_3), 94.05 (CH-8), 99.13 (CH-6), 103.19 (C), 114.83 (C), 150.67 (C), 154.84 (C), 157.43 (C), 160.09 (C), 163.24 (C=O).

4-Methyl-5,7-dihydroxycoumarin (15)
^{1}H NMR (400 MHz, MeOD): δ 2.60 (3H, s, Me), 5.85 (1H, s, H-3), 6.23 (2H, bs, H6-8).
^{13}C NMR (100 MHz, MeOD): δ 22.72 (CH_3), 94.41 (CH-8), 98.91 (CH-6), 102.71 (C), 108.22 (CH-3), 156.67 (C), 156.89 (C), 158.18 (C), 161.56 (C), 162.64 (C=O).

3,4-Dimethyl-7-hydroxycoumarin (16)
^{1}H NMR (400 MHz, MeOD): δ 2.15 (3H, s, Me), 2.41 (3H, s, Me), 6.69 (1H, d, *J*= 2.4 Hz, H-8), 6.82 (1H, dd, *J*= 2.4; 8.8 Hz, H-6), 7.60 (1H, d, *J*= 8.8 Hz, H-5).
^{13}C NMR (100 MHz, MeOD): δ 11.53 (CH_3), 13.68 (CH_3), 101.72 (CH-8), 112.71 (CH-6), 113.02 (C), 117.04 (C), 125.65 (CH-5), 147.93 (C), 153.43 (C), 160.29 (C), 163.19 (C=O).

4-Methyl-7-hydroxycoumarin (17)
^{1}H NMR (400 MHz, MeOD): δ 2.42 (3H, s, Me), 6.09 (1H, s, H-3), 6.69 (1H, bs, H-8), 6.82 (1H, bs, H-6), 7.57 (1H, bs, H-5).
^{13}C NMR (100 MHz, MeOD): δ 17.28 (CH_3), 102.08 (CH-8), 109.81 (CH-3), 112.41 (C), 112.94 (CH-6), 125.95 (CH-5), 154.49 (C), 155.08 (C), 161.57 (C), 162.46 (C=O).

Biological studies: All starting materials were commercially available, research-grade chemicals, and were used without further purification. RPMI 1640 medium was purchased from Sigma-Aldrich (St Louis, MO, USA), fetal bovine serum (FBS) from Nataclor (Nataclor-Argentina), trichloroacetic acid (TCA) and dimethylsulfoxide (DMSO) from Merck (Darmstadt, Germany), and penicillin G-streptomycin, sulforhodamine B (SRB) and glutamine from Sigma-Aldrich (St Louis, MO, USA). Pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration, that is, 100, 10 and 1 μM.

Antiproliferative assay: The human solid tumor cell lines HeLa (cervix), T-47D (breast) and WiDr (colon) were used in this study. Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine in a 37ºC, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and re-suspended in antibiotic containing medium (100 U penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated at 100 μL per well at densities of 1.5 x 10^4 (HeLa and T-47D), and 2 x 10^4 (WiDr) cells per well, based on their doubling times. Each agent was tested at least in triplicate at different dilutions in the range of 1–100 μM. The drug treatment was started on day 1 after plating. Drug incubation time was 48 h, after which time cells were precipitated with 25 μL ice-cold TCA (50%, w/v) and fixed for 60 min at 4ºC.

Then the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing medium.

The percentage of growth (PG) was calculated with respect to untreated control cells (*C*) at each of the drug concentration levels based on the difference in OD at the start (*T0*) and end of drug exposure (*T*), according to the National Cancer Institute (USA) formulas 0. Therefore, if *T* is greater than or equal to *T0* the calculation is PG = 100 [(*T*-*T0*)/(*C*-*T0*)]. If *T* is less than *T0* denoting cell killing the calculation is PG = 100 [(*T*-*T0*)/(*T0*)].

With these calculations, 3 levels of effect could be determined; 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell killing (LC_{50}) that represent the concentration at which PG is +50, 0, and -50, respectively. Thus, a PG value of 0 corresponds to the amount of cells present at the start of drug exposure, while negative PG values denote net cell kill.

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Accounts/Reviews

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Contents

Original Paper

Continued inside backcover