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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of prenyloxy coumarin analogues and evaluation of their antioxidant, lipoxygenase (LOX) inhibitory and cytotoxic activity

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Abstract Umbelliferone, 4-methyl-umbelliferone and their farnesyloxy and geranyloxy analogues were synthesized and evaluated for their antioxidant and soybean lipoxygenase inhibitory activity as well as their cytotoxicity against human neuroblastoma cell line SK-N-SH and human hepatoma cell line HepG2. Auraptene (**3**), followed by 4-methyl-auraptene (**4**) exhibit modest cytotoxity against the HepG2 cell line. The novel coumarin **6** combines a satisfactory lipoxygenase inhibitory activity with potent cytotoxicity against SK-N-SH cells, but not against HepG2 cells, thus it could be considered as a lead compound for the development of novel therapeutic agents against neuroblastoma.

Keywords Coumarin · Umbelliprenin · Auraptene · Lipoxygenase · Cytotoxicity

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Introduction

Coumarins are heterocyclic organic compounds widely distributed in the plant kingdom. They exhibit important biological properties including antioxidant, anticancer, vasorelaxant, antiviral and anti-inflammatory activities (Roussaki et al. 2010, 2014).

Umbelliferone (**1**) (Fig. 1) is a natural coumarin encountered in numerous plants (Vialart et al. 2012) which presents remarkable antiparasitic, anticancer and antioxidant activities. (Chiang et al. 2010; Venugopala et al. 2013; Ramalingam and Vaiyapuri 2013; Singh et al. 2010).

4-Methyl-umbelliferone (**2**), is a 7-hydroxy-coumarin obtained from umbelliferous plants (Apiaceae), including anise, cumin, parsley, and dill (Kultti et al. 2009; García-Vilas et al. 2013). 4-Methyl-umbelliferone exhibits a variety of activities including antioxidant, anticancer (Yates et al. 2015) and antibacterial (Fang et al. 2014). Additionally, 4-methyl-umbelliferone (**2**) behaves as an antiangiogenic compound (García-Vilas et al. 2013).

Oxyprenylated products (isopentenyloxy-, geranyloxy- and the less encountered farnesyloxy-compounds and their biosynthetic derivatives) represent a family of secondary metabolites that have been considered for years just as biosynthetic intermediates of C-prenylated derivatives. Only in the last decade these natural products have been recognized as interesting and valuable biologically active phytochemicals (Alhassan et al. 2014).

Chemical modification such as prenylation, enhances the structural variety of coumarins and as a result these natural products represent nowadays a new frontier for the development of novel drugs (Roussaki et al. 2014). Prenyloxycoumarins are secondary metabolites commonly present in plants belonging to the families of Rutaceae and Umbelliferae. Several of these coumarins were shown to

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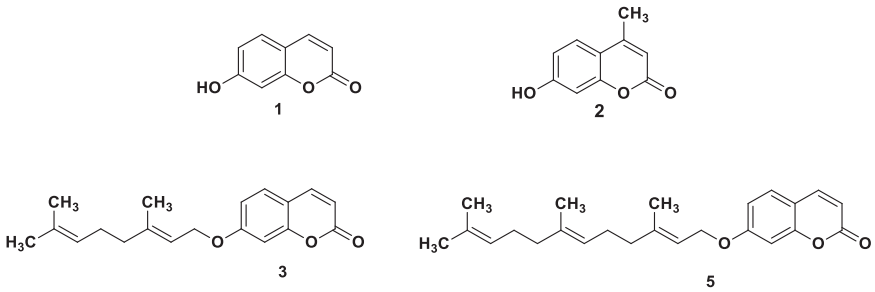


Fig. 1 Chemical structures of umbelliferone (**1**), 4-methyl-umbelliferone (hymechromone) (**2**), Auraptene (**3**), Umbelliprenin (**5**)

possess valuable pharmacological properties. Among them, auraptene (7-geranyloxycoumarin, **3**) which was first isolated from the peel of citrus fruit (*Citrus natsudaidai* Hayata) (Kariyone and Matsuno 1953) has been reported to have chemopreventive effects on chemically induced carcinogenesis (Saldanha et al. 1990). Auraptene (**3**) is a promising chemopreventive agent against skin (Murakami et al. 1997), tongue, esophagus, and colon carcinogenesis in rodents (Soltani et al. 2010) and can also modulate fat metabolism (Takahashi et al. 2008). In addition, auraptene inhibits the production of tumor necrosis factor alpha (TNF-α) in LPS-stimulated RAW264.7 macrophages (Banbury et al. 2015). Recently, auraptene was tested as β-lactamase inhibitor, which is the main cause of resistance against β-lactam antibiotics, and displayed the most potent inhibitory activity toward class A β-lactamase among the other tested compounds (Safdari et al. 2014).

Another natural oxyprenylated coumarin is umbelliprenin (7-farnesyloxy-coumarin, **5**). Umbelliprenin (**5**) synthesized by various Ferula plant species (Iranshahi et al. 2009) has been studied for its anticancer activity in different cancer cell lines (Barthomeuf et al. 2008; Khaghanzadeh et al. 2012; Valiahdi et al. 2013; Jun et al. 2014; Zhang et al. 2015). Umbelliprenin (**5**) has also been shown to exhibit an inhibitory effect on the activity of matrix metalloproteinases, which play critical roles in cancer metastatic cascade, such as migration, angiogenesis, and invasiveness (Shahverdi et al. 2006). In addition, various in vitro and in vivo models have provided evidence of the antioxidant, anti-inflammatory (Zamani Taghizadeh Rabe et al. 2016; Iranshahi et al. 2009) and cancer chemopreventive properties of umbelliprenin (Iranshahi et al. 2008). The structures of coumarins **1, 2, 3, 5** are presented in Fig. 1.

Lipoxygenases (LOX) are iron-containing enzymes widely distributed in plants and animals. They catalyze the oxidation of polyunsaturated fatty acids such as linoleic acid (in plants) and arachidonic acid (in mammals) at specific positions to hydroperoxides. In humans LOX plays a key role in the biosynthesis of leukotrienes (LT's), the proinflammatory mediators mainly released from myeloid cells. Arachidonic acid is converted to LTA 4 by the enzyme 5-lipoxygenase. LTA 4 is an unstable intermediate which after a process of continuing conversion results in the formation of LT's (Detsi et al. 2009). Thus, inhibitors of LOX have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, but their therapeutic potential has now been expanded to certain types of cancer, cardiovascular diseases, acute and chronic inflammation, asthma and rhinitis, arthritis, psoriasis, and hereditary ichthyosis (Haeggstrom and Funk 2011). The majority of LOX inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs via a carbon centered radical, and these compounds can inhibit the formation of the radical or trap it once formed (Iranshahi et al. 2009; Kallitsakis et al. 2014; Pontiki and Hadjipavlou-Litina 2007).

As a continuation of our studies concerning the biological activity of coumarin analogues (Roussaki et al. 2010, 2014) we present here the synthesis and structural characterization of a series of natural and non-natural coumarins, and the evaluation of their in vitro antioxidant, soybean LOX inhibitory activities and cytotoxic activity against human neuroblastoma cell line SK-N-SH and human hepatoma cell line HepG2.

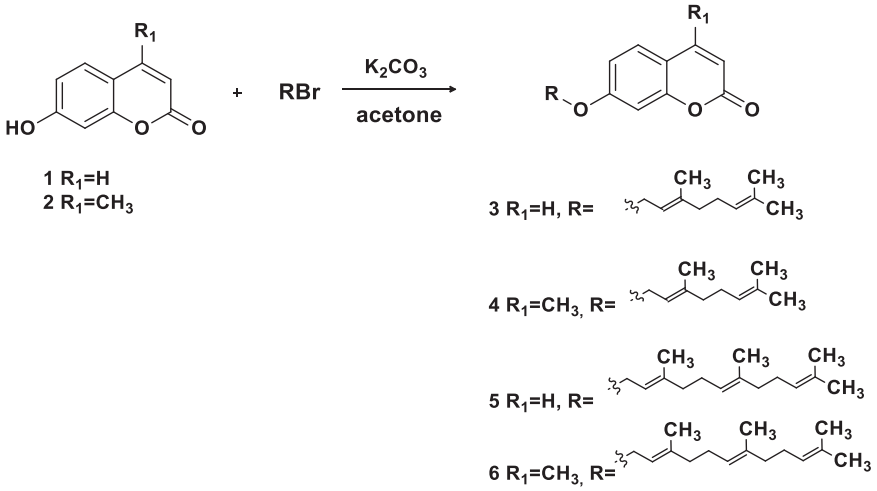
Results and discussion

Chemistry

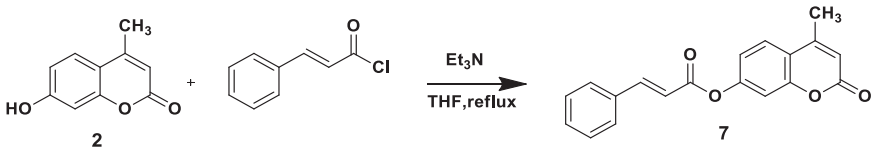
In order to efficiently synthesize the desired prenyloxy-coumarins **3–6**, umbelliferone (**1**, commercially available) and 4-methyl-7-hydroxy-coumarin (**2**, synthesized via Pechmann condensation between resorcinol and ethyl acetoacetate in the presence of concentrated H_2SO_4) (Kiskhan and Yagci 2007) were used as starting materials (Scheme 1). Alkylation of these coumarins with geranyl and farnesyl bromide in a basic environment, provided compounds **3–6** in good yields and high purity (Askari et al. 2009). The structures of the synthesized compounds were identified using NMR spectroscopy.

The study of compounds that encompass two or more pharmacophores on the same structural framework, is a well-established approach in medicinal chemistry. Phenolic acids, such as cinnamic, ferulic, and caffeic acid, account for an important part of the antioxidant activity displayed by

Scheme 1 Synthesis of prenyloxy-coumarins **3–6**



Scheme 2 Synthesis of coumarin **7**



fruits, vegetables and several beverages such as juices, wine and beer together with flavonoid polyphenol phytochemicals (Piazzon et al. 2012). Investigation of the potential of phenolic acids as phytoprotectants revealed that they possess important anticancer (Gomes et al. 2003; Rocha et al. 2012), cardioprotective (Spilioti et al. 2014) and anti-inflammatory activity (Leal et al. 2011). Moreover, several compounds bearing the cinnamate functionality have been recently shown to potently inhibit soybean LOX activity (Pontiki et al. 2014; Peperidou et al. 2014). Thus, we decided to synthesize the ester of cinnamic acid with 4-methyl-7-hydroxy-coumarin (**2**) in order to investigate the impact of this structural feature on the biological activity of coumarins. The synthesis was straightforward, and involved the reaction between cinnamoyl-chloride and coumarin **2**, in the presence of triethylamine using tetrahydrofuran (THF) as a solvent (Scheme 2). The desired ester **7** was obtained as a white solid without the need of further purification as shown by NMR spectroscopy.

Antioxidant activity evaluation

Taking the multifactorial character of oxidative stress into account, we decided to evaluate the in vitro antioxidant activity of the synthesized molecules using four different antioxidant assays. Therefore, the radical scavenging ability of the compounds was tested against the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical and the competition of the compounds with DMSO for $HO^{\bullet}$, generated by the Fe^{3+}/ascorbic acid system, expressed as percent inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. Finally, the ability of the synthesized coumarins to inhibit lipid peroxidation (LP) induced by the thermal free radical producer 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was evaluated whereas generation of the $ABTS^{\bullet+}$ (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) radical cation, formed the basis for a further determination of the antioxidant activity of our compounds (Pontiki et al. 2009, 2011; Roussaki et al. 2010, 2014). The results are presented in Table 1.

The tested coumarin analogues **1–7** do not possess satisfactory DPPH radical scavenging ability. This observation could be attributed firstly to the absence of phenolic hydroxyl groups on the tested compounds and secondly to the presence of bulky substituents such as the geranyl and farnesyl moieties which could prevent effective interaction of the compounds with the DPPH radical due to steric hindrance.

The measured inhibition of LP showed that six out of the seven tested analogues exhibited significant LP inhibition, higher than the reference compound trolox. The most efficient LP inhibitor is 4-methyl-umbelliferone (**2**) (93%), followed by umbelliferone (**1**) and umbelliprenin (**5**). The presence of a 4-methyl substituent on umbelliprenin, leads to a less active compound (**6**) (47%). Auraptene (**3**) displays higher activity than its 4-methyl analogue (**4**). In addition, auraptene (**3**) and 4-methyl-auraptene (**4**) exhibit lower

Table 1 Interaction % with DPPH, % inhibition of lipid peroxidation (LP) induced by AAPH, % $HO^{\bullet}$ radical scavenging ability, $ABTS^{\bullet+}$ decolorization ($ABTS^{\bullet+}$ %) and inhibition of soybean lipoxygenase (LOX) % at 100 μM or IC_{50}) for compounds **1–7**

Compound	clog P (Biobyte Corp. C-QSAR Database)	DPPH scavenging ability (%) (100 μM)		% Inhibition of LP induced by AAPH (100 μM)	$HO^{\bullet}$ (%) (100 μM)	$ABTS^{\bullet}$+ (%) (100 μM)	Inhibition of soybean lipoxygenase	
		20 min	60 min				% (100 μM)	IC50 (μM)
1	1.58	1	2	92	n.t.	n.t.	100	41
2	2.12	No	No	93	n.t.	n.t.	No	
3	5.48	26	6	52	100	no	44	
4	5.98	21	No	20	49	79	78	65
5	7.51	45	47	92	n.t.	n.t.	4	
6	8.01	31	4	47	100	89	77	76
7	4.29	22	2	68	83	75	50	100
NDGA		84	83	–			86	4.5
Trolox		–	63	73		–		
Ascorbic acid					96			

n.t. not tested, *NDGA* Nordihydroguaiaretic acid

inhibition of LP than the corresponding 7-hydroxy analogues **1** and **2**, indicating that the presence of the geranyloxy-moiety does not favor this activity. Esterification of the free hydroxyl-group of 4-methyl-umbelliferone with cinnamic acid leads to compound **7** with satisfactory LP inhibitory activity (68%), albeit lower than the starting 4-methyl-umbelliferone (**2**).

Hydroxyl radicals are among the most reactive oxygen species and are considered to be in part responsible for tissue damage occurring in inflammation (Pontiki and Hadjipavlou-Litina 2007). Auraptene (**3**) and 4-methyl-umbelliprenin (**6**) are very effective $HO^{\bullet}$ scavengers (100%) whereas ester **7** also shows satisfactory activity against this free radical.

Coumarin **6** is the most potent $ABTS^{\bullet+}$ radical cation scavenger among the compounds tested in this work, followed by 4-methyl-auraptene (**4**) and ester **7**.

Anti-inflammatory activity evaluation

Furthermore, the ability of the compounds to inhibit soybean LOX, using the UV absorbance based enzyme assay, was determined as an indication of potential anti-inflammatory activity (Kontogiorgis and Hadjipavlou-Litina 2003). The results are presented in Table 1. Although the results of this assay cannot be extrapolated to the inhibition of mammalian 5-LOX, it has been shown that inhibition of plant LOX activity by nonsteroidal anti-inflammatory agents is qualitatively similar to the inhibition they cause to the rat mast cell LOX. Therefore, the soybean inhibition assay can be used as a simple qualitative assay for such activity.

The most active LOX inhibitor among the tested coumarin derivatives is umbelliferone (**1**) followed by 4-methyl-auraptene (**4**) (41 μM, 65 μM, respectively) and 4-methyl-umbelliprenin (**6**) (IC_{50} 76 μM).

It is noteworthy that insertion of a methyl group at position 4 of the umbelliferone structure (compound **2**) leads to complete loss of activity whereas in the case of auraptene (**3**), the presence of methyl group enhanced the LOX inhibitory activity (compound **4**). Respectively, the novel compound 4-methyl-umbelliprenin (**6**) is a better inhibitor of LOX in comparison with umbelliprenin (**5**). Cinnamic ester (**7**) displays lower activity as inhibitor of LOX (IC_{50} 100 μM). Although lipophilicity is a major physicochemical property influencing activity, the presented results do not support this idea. On the contrary steric effects seem to be more significant.

Glutathione conjugation

Glutathione (GSH) conjugation is an important pathway by which reactive electrophilic compounds are detoxified. It protects vital cellular constituents against chemical reactive species by virtue of its nucleophilic sulfhydryl group and constitutes an in vivo antioxidant protective mechanism. The nucleophilic addition of GSH to electron-deficient carbon double bonds occurs mainly in compounds with α,β-unsaturated double bonds. In most instances the double bond is rendered electron deficient by resonance or conjugation with a carbonyl group. The coumarins synthesized in this study were tested for their ability to react with GSH in vitro and the results are presented in Table 2.

Table 2 Study of the conjugation of coumarins **3, 4, 6** and **7** with GSH

Compound	λ_{max}	ε_{max}
3	325	136
3 + 2GSH	325	148
3 + 10GSH	325	143
4	322	516
4 + 2GSH	322	738
4 + 10GSH	322	715
6	322	261
6 + 2GSH	322	384
6 + 10GSH	322	359
7	329	318
7 + 2GSH	329	543
7 + 10GSH	329	368

GSH Glutathione

No conjugation was observed between the tested compounds and GSH. This was supported by the determined ε_{max} values at λ_{max}.. It is most likely that stereochemistry affects the conjugation process.

Cytotoxic activity evaluation

Coumarins have been reported to exhibit toxicity against different types of cancer cells. The investigation of potential cytotoxicity by our compounds was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the human neuroblastoma cell line SK-N-SH and the human hepatoma cell line HepG2 (Berridge and Tan 1993) The results are presented in Figs. 2 and 3.

The natural coumarin auraptene (**3**) is the most potent cytotoxic coumarin among the studied compounds in both cell lines. The farnesyloxy coumarin umbelliprenin (**5**) shows cytotoxicity only on the SK-N-SH cell line whereas it is not cytotoxic on HepG2 cells. 4-methyl-auraptene (**4**) possesses decreased cytotoxic activity on both cell lines as compared to auraptene (**3**). In the case of 4-methyl-umbelliprenin (**6**) the cytotoxicity on SK-N-SH cells was similar to that of umbelliprenin (**5**), however the compound shows a moderate cytotoxicity on HepG2 cells.

Umbelliferone (**1**) and hymechromone (**2**) affect cell viability only at high concentrations thus, as far as cytotoxicity against SK-N-SH and HepG2 cells is concerned, specific alkylation of the 7-OH group significantly enhances cytotoxic activity. On the other hand, esterification of the 7-OH of hymechromone with cinnamic acid leads to a compound which is not cytotoxic on either cell line (coumarin analogue **7**).

Conclusions

In conclusion, umbelliferone, hymechromone and their farnesyloxy and geranyloxy analogues as well as the ester of cinnamic acid with 7-hydroxy-4-methyl-coumarin, were synthesized and tested for their antioxidant and cytotoxic activity. The results of this study indicate that umbelliferone (**1**), 4-methyl-auraptene (**4**) and the new analogue 4-methyl-umbelliprenin (**6**) efficiently inhibit soybean LOX while umbelliferone (**1**) is also a potent LP inhibitor. Auraptene (**3**) exhibits the most potent cytotoxic activity, followed by 4-methyl-auraptene (**4**), umbelliprenin (**5**) and 4-methyl-umbelliprenin (**6**) against the SK-N-SH cell line thus they could be considered as drug candidates for neuroblastoma therapy after additional evaluation. Auraptene (**3**), followed by 4-methyl-auraptene (**4**) exhibit modest cytotoxity against the HepG2 cell line, whereas all other coumarin analogs are not cytotoxic against HepG2. Overall, as it has been shown that inhibition of the leukotriene pathway leads to apoptosis of neuroblastoma cells in vitro (Sveinbjornsson et al. 2008) the novel coumarin **6**, which combines a satisfactory LOX inhibitory activity with potent cytotoxicity against the SK-N-SH line and a very significant antioxidant profile, should be further explored as a lead compound for the development of novel therapeutic agents against neuroblastoma.

Experimental

Chemicals and instruments

The chemicals used for synthesis and analysis were purchased from Sigma-Aldrich or Alfa Aesar (7-hydroxycoumarin, 98%) and used without further purification. NMR spectra were recorded on a Varian 300 MHz spectrometer at the National Hellenic Research Foundation. The HR-MS spectrum was obtained using a UHPLC-MSn Orbitrap Velos-Thermo mass spectrometer. Melting points were determined on a Gallenkamp MFB-595 melting point apparatus and are uncorrected.

Synthesis of 4-methyl-7-hydroxy coumarin (4 methyl umbelliferone) (2)

The desired compound was prepared following the method of Kiskhan and Yagci (Kiskhan and Yagci 2007) Yield: 65%; m.p.:186–187 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 10.47 (s, 1H, 7-OH), 7.56 (d, $J = 8.7$ Hz, 1H, H-5), 6.77 (dd, $J_{6,8} = 1.8$ Hz, $J_{6,5} = 8.4$ Hz, 1H, H-6), 6.68 (d, $J_{8,6} = 1.8$ Hz, 1H, H-8), 6.10 (s, 1H, H-3), 2.36 (s, 3H, 4-CH_3).

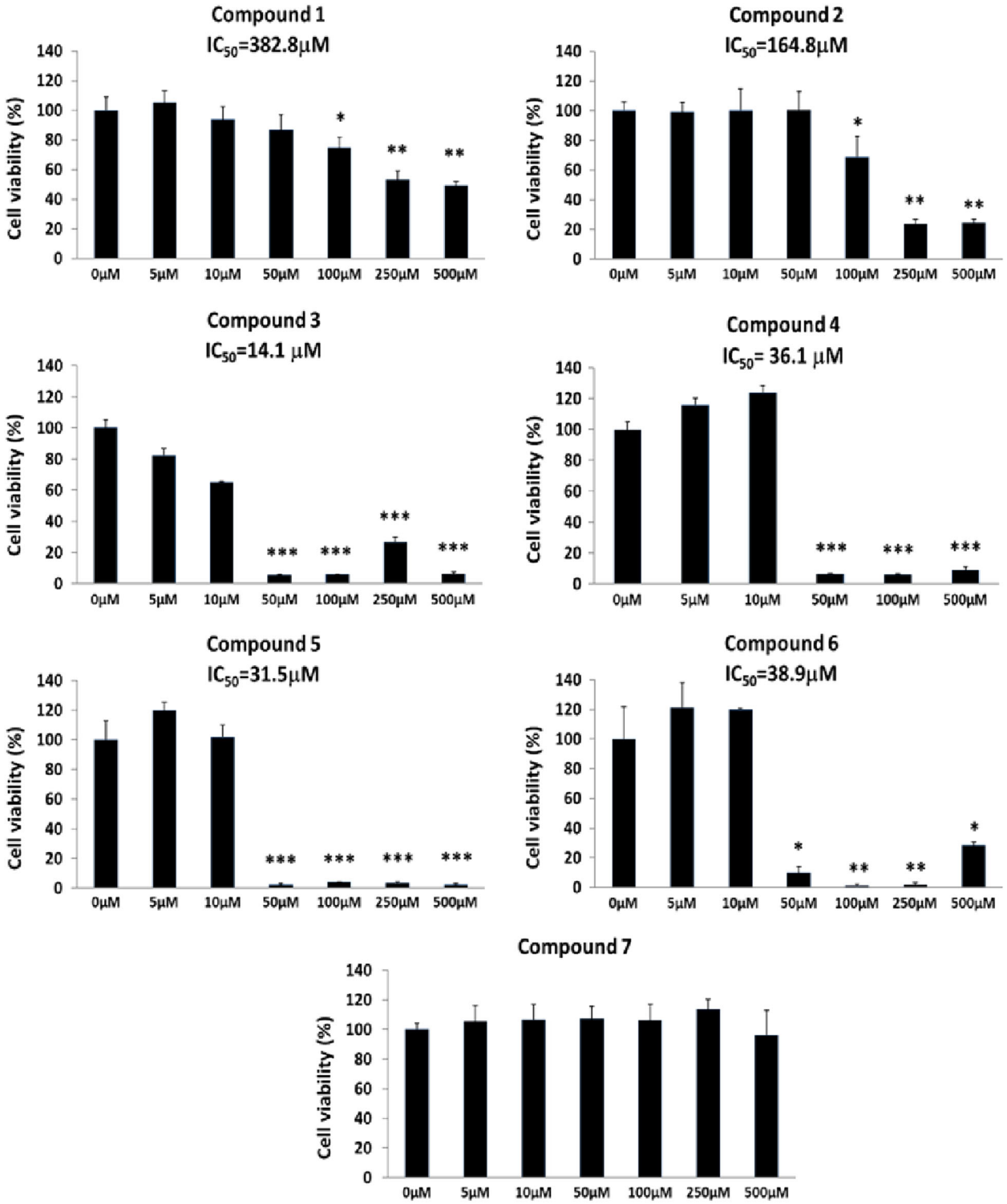


Fig. 2 Effect of coumarin analogues on human neuroblastoma cell SK-N-SH viability. Shown is the survival of SK-N-SH cells incubated with coumarin analogs at various concentrations for 48 h, as determined by a MTT assay. Cell viability is expressed as percent relative to the viability of control untreated cells (0 μM, incubation with the compounds dilution medium) set to 100%. Data are the means ± SD $*p < 0.05$ vs. control; $**p < 0.005$ vs. control; $***p < 0.0005$ vs. control

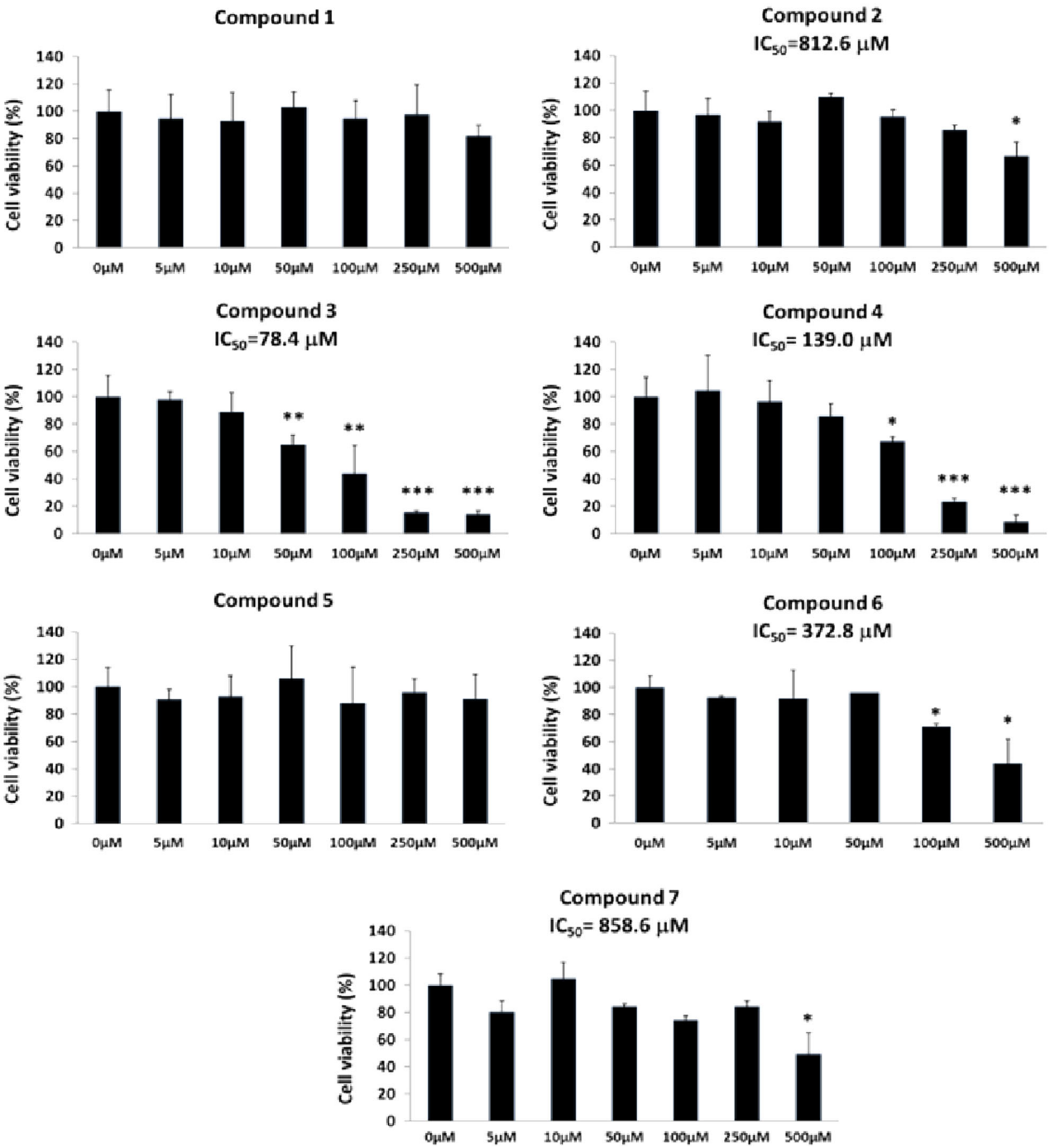


Fig. 3 Effect of coumarin analogues on human hepatoma cell line HepG2 viability. Shown is the survival of HepG2 cells incubated with coumarin analogs at various concentrations for 24 h, as determined by a MTT assay. Cell viability is expressed as percent relative to the viability of control untreated cells (0 μM, incubation with the compounds dilution medium) set to 100%. Data are the means ± SD $*p < 0.05$ vs. control; $**p < 0.005$ vs. control; $***p < 0.0005$ vs. control

General procedure for the synthesis of prenyloxy-coumarins ***3–6***

A mixture of the appropriate 7-hydroxy-coumarin and farnesyl or geranyl-bromide in acetone in the presence of potassium carbonate (K_2CO_3) was refluxed overnight. After the completion of the reaction, potassium carbonate was filtrated, the precipitate was washed with acetone and the solvent was removed under reduced pressure. The products were purified by recrystallization.

7-geranyloxy-coumarin (auraptene) (**3**) was prepared following the general procedure, starting from 0.0015 mol

(0.25 g) 7-hydroxy-coumarin (**1**), 0.002 mol (0.4 mL) geranyl bromide and 0.0015 mol (0.21g) K_2CO_3 in 21.6 mL acetone. The white solid product was collected after recrystallization from methanol and hexane. Yield: 52%, m.p.: 66–67 °C (lit. m.p. 62.7–63.4 °C) (Askari et al. 2009). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.63 (d, $J = 9.3$ Hz, 1H, H-4), 7.36 (d, $J = 8.4$, 1H, H-5), 6.86–6.82 (m, 2H, H-6 & H-8), 6.24 (d, $J = 9.3$ Hz, 1H, H-3), 5.47 (t, $J = 6$ Hz, 1H, H-2′), 5.09 (t, $J = 5.7$ Hz, 1H, H-6′), 4.62 (d, $J = 6.6$ Hz, 2H, H-1′), 2.15-2.11 (m, 4H, H-4′ & H-5′), 1.79 (s, 3H, 3′-CH_3), 1.69 (s, 3H, 8′-CH_3), 1.63 (s, 3H, 7′-CH_3).

4-methyl-7-geranyloxy-coumarin (4-methyl-auraptene) (**4**): Prepared following the general procedure, starting from 0.0014 mol (0.25 g) 4-methyl-7-hydroxy- coumarin (**2**), 0.0015 mol (0.3 mL) geranyl bromide and 0.0014 mol (0.19 g) of K_2CO_3 in 19.9 mL acetone. The brown gummy solid product was collected after recrystallization from methanol (3 mL) and hexane (1 mL). Yield: 68%, ^{1}H NMR (300 MHz,DMSO-d_6): δ (ppm) 7.64 (d, $J = 8.4$ Hz, 1H, H-5), 6.94–6.91 (m, 2H, H-6 & H-8), 6.18 (s, 1H, H-3), 5.42 (t, $J = 6$ Hz, 1H, H-2'), 5.03 (br, 1H, H-6′), 4.65 (d, $J = 6.6$ Hz, 2H, H-1′), 2.39 (s, 3H, 4-CH_3), 2.08–2.06 (m, 4H, H-4′ & H-5′), 1.73 (s, 3H, 3′-CH_3), 1.61 (s, 3H, 8′-CH_3), 1.56 (s, 3H, 7′-CH_3).

7-farnesyloxy- coumarin (Umbelliprenin) (**5**): Prepared following the general procedure, starting from 0.0015 mol (0.25 g) 7-hydroxy- coumarin (**1**), 0.00185 mol (0.5 mL) farnesyl bromide and 0.0015 mol (0.21g) K_2CO_3 in 21.6 mL acetone. The yellow solid product was collected after recrystallization from methanol (3 mL) and hexane (1 mL). Yield: 30%, m.p.: 61–63 °C (lit. m.p.: 57.5–59.1 °C) (Askari et al. 2009). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.62 (d, $J = 9.3$ Hz, 1H, H-4), 7.35 (d, $J = 8.1$ Hz, 1H, H-5), 6.86-6.82 (m, 2H, H-6 & H-8), 6.24 (d, $J = 9.6$ Hz, 1H, H-3), 5.48 (t, $J = 6.6$ Hz , 1H, H-2′), 4.62 (d, $J = 6.6$ Hz , 1H, H-1′), 2.18 -2.01 (m, 8H, H-4′ & H-5′ & H-8′ & H-9′), 1.79 (s, 3H, 3′-CH_3), 1.70 (s, 3H, 8′-CH_3), 1.63 (s, 6H, 11′-CH_3 & 12′-CH_3).

4-methyl-7-farnesyloxy-coumarin (4-methyl-Umbelliprenin) (**6**): Prepared following the general procedure, starting from 0.00142 mol (0.25 g) 4-methyl-7-hydroxy-coumarin (**2**), 0.0017(0.46 mL) farnesyl bromide and 0.00142 mol (0.196 g) K_2CO_3 in 19.9 mL acetone. The yellow gummy solid product was collected after recrystallization from methanol and hexane. Yield: 50%.^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.48 (d, $J = 8.7$ Hz, 1H, H-5), 6.86 (d, $J_{6,8} = 1.8$ Hz, $J_{6,5} = 8.7$ Hz, 1H, H-6), 6.82 (d, $J_{8,6} = 1.8$ Hz, 1H, H-8), 6.13 (s, 1H, H-3) , 5.47 (t, $J = 6.9$ Hz, 1H, H-2′), 5.10- 5.07 (m, 1H, H-6′), 4.60 (d, $J = 6.6$ Hz, 1H, H-1′), 2.39 (s, 3H, 4-CH_3), 2.12–1.95 (m, 8H, H-4′ & H-5′ & H-8′ & H-9′), 1.76 (s, 3H, 3′-CH_3), 1.67 (s, 3H, 8′-CH_3), 1.59 (s, 6H, 11′-CH_3 & 12′-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 161.95 (C-2), 160.59 (C-7), 155.14 (C-8a), 153.85 (C-4), 141.46 (C-4), 135.14 (C-3′), 131.06 (C-7′), 126.79 (C-11′), 124.52 (C-5), 123.89 (C-6′ & C-10′), 119.52 (C-2′), 113.45 (C-4a), 113.05 (C-6), 111.48 (C-3), 101.82 (C-8), 65.55 (C-1′), 39.67 (C-8′), 39.30 (C-4′), 26.58 (C-9′), 25.99 (C-5′), 25.92 (12′-CH_3), 18.56 (4-CH_3), 17.96 (3′-CH_3), 16.85 (8”-CH_3), 16.27 (11′-CH_3). HRMS calcd for $C_{25}H_{32}O_3$ Na: *m/z*: 403.2244, found: 403.2245.

*4-methyl-2-oxo-2H-chromen-7-yl cinnamate (**7**)*

0.00142 mol (0.25 g) 4-methyl-7-hydroxy-coumarin (**2**), 0.00213 mol (0.356 g) cinnamoyl chloride, and 5.5 mL triethylamine (Et_3N) were added in 7.1 mL dry THF and stirred for 24 h. After the reaction is adding a suitable amount of water, followed by extracting the mixture with dichloromethane (CH_2Cl_2) The organic phase was dried over anhydrous sodium sulfate (Na_2SO_4), filtered and evaporated to give the final white solid product in high purity. Yield: 77%, m.p.: 145–146 °C (lit. m.p.: 149–151 °C) (Nakayama and Kanaoka 1973). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.91 (d, $J = 15.9$ Hz, 1H, H-b), 7.65-7.59 (m, 3H, H-6′ & H-2′), 7.45-7.43 (m, 3H, H-3′ & H-5′ & H-4′), 7.21 (d, $J = 2.1$ Hz, 1H, H-8), 7.17 (dd, $J = 2.1$ Hz, $J = 8.4$ Hz, 1H, H-6), 6.65 (d, $J = 15.9$ Hz, 1H, H-a), 6.29 (s, 1H, H-3), 2.47 (s, 3H, 4-CH_3).

Determination of the reducing activity of the stable radical DPPH

To an ethanolic solution of DPPH (100 µM) in absolute ethanol an equal volume of the compounds dissolved in DMSO was added (100 µM). The mixture was shaken vigorously, in some cases with the help of ultrasound, and then allowed to stand for 20 or 60 min; absorbance at 517 nm was determined spectrophotometrically and the percentage of activity was calculated. All tests were undertaken on three replicates and the results were averaged and compared with the appropriate standard NDGA (Table 1) (Hadjipavlou et al. 2009).

Inhibition of linoleic acid lipid peroxidation

Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234 nm. AAPH is used as a free radical initiator. Ten microliters of the 16 mM linoleic acid sodium salt solution was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 µL of 40 mM AAPH solution. Oxidation was carried out in the presence of coumarins (10 µL, from a stock solution of 10 mM in DMSO) in the assay. Lipid

oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides and compared with the appropriate standard trolox (Hadjipavlou et al. 2009).

Competition of the tested compounds with DMSO for hydroxyl radicals

The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system, were detected by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 mM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds and ascorbic acid (10 mM). After 30 min of incubation at 37 °C the reaction was stopped with CCl_3COOH (17% w/v) and the % competition of the tested compounds with DMSO for hydroxyl radicals was determined (Table 1) (Pontiki and Hadjipavlou-Litina 2006).

$ABTS^{\bullet+}$—decolorization assay for antioxidant activity

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12e16 h before use. For the present study, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Stock solutions of the tested compounds in DMSO were diluted so that, after introduction of a 10 mL aliquot of each dilution into the assay, they produced between 20 and 80% inhibition of the blank absorbance. After addition of 1.0 mL of diluted $ABTS^{\bullet+}$ solution ($\lambda = 734$ nm) to 10 mL of antioxidant compounds or Trolox standards (final concentration 0–0.1 mM) in ethanol the absorbance reading was taken at 30 °C exactly 1 min after the initial mixing (Table 1).

Soybean LOX inhibition study in vitro

The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 ml) and 0.2 ml of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor NDGA (Hadjipavlou et al. 2009).

Glutathione conjugation assay

Stock solutions of the compounds were prepared in water using phosphates buffer solution (PBS) pH 7.4 and in order to achieve dissolution the solvent contained approximately 10% DMSO. The concentrations of the solutions were chosen so that the absorption maxima were between 0.5 and 1. The test compounds are incubated for 24 h at 37 °C and their UV spectra were recorded. All determinations were carried out in duplicate. The error limits of the ε values were approximately 2%. The experiment was repeated in the presence of GSH using thiol/test compound, 2/1 and 10/1 and incubation at 37 °C for 24 h and their UV spectra were recorded. (Pontiki and Hadjipavlou-Litina 2007).

Cell viability assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay (Berridge and Tan 1993). Human neuroblastoma SK-N-SH cells (ATCC) were cultured in minimal essential medium (MEM) Earle's supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/liter sodium bicarbonate, 10% FBS and antibiotics. Human hepatoma HepG2 cells (ATCC) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (high glucose), 10% FBS and antibiotics. The cells were seeded in 96 well plates at a density of 2×10^4 cells/well in complete cell culture medium. The coumarin compounds were dissolved in DMSO and then diluted at the appropriate concentration in the culture medium. The final DMSO concentration in each well was 0.5% (v/v). Twenty four hours after seeding, the media were changed into serum-free medium containing the compounds, at increasing concentrations of 5, 10, 50, 100, 250, and 500 μM, and the cells were incubated for 24 h (HepG2) or 48 h (SK-N-SH) at 37 °C. At the end of the incubation, the cells were further incubated in serum-free medium containing 0.65 mg/ml MTT for 3 h at 37 °C. Then, the medium was removed, DMSO was added in each well to dissolve the dark blue formazan crystals formed by metabolically active cells containing functional mitochondria and absorbance was read at 570 nm. At least two independent experiments were done in triplicate for each compound and the IC_{50} values were calculated using the GraphPad Prism software.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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