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Synthesis and biological evaluation of hydroxylated 3-phenylcoumarins as antioxidants and antiproliferative agents

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ABSTRACT

Based on the observed biological activities of coumarins and resveratrol, we synthesized fourteen hydroxylated 3-phenylcoumarins (stilbene-coumarin hybrids) including six novel *ortho*-hydroxy-methoxy substituted derivatives, **1**–**14**, by Perkin reaction. We characterized these compounds concerning their antioxidant activity against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced pBR322 DNA strand breakage, and their antiproliferative effects on human promyelocytic leukemia HL-60 and human lung adenocarcinoma epithelial A549 cells. Structure–activity relationship information suggests that the introduction of *ortho*-hydroxy-methoxy groups and *ortho*-dihydroxy groups on the aromatic A ring could efficiently improve antiproliferative activity. Interestingly, a new derivative, 6-methoxy-7-hydroxy-3-(4′-hydroxyphenyl)coumarin, **9**, behaved as a poor antioxidant but appeared to be the most potent antiproliferative agent among the compounds examined, and this activity was mediated by deregulation in cell cycle and induction of apoptosis.

Coumarins (1,2-benzopyrones) are an important family of naturally occurring compounds, and are being extensively investigated because of their wide rage of biological activities, such as anticancer,[1] enzyme inhibition,[2,3] vasorelaxant,[4] antimicrobial,[5,6] antioxidant,[7] and anti-inflammatory.[4,7] and anti-HIV.[8] Most of coumarins are C7-hydroxylated in nature.[9,10] Especially, scopoletin (6-methoxy-7-hydroxycoumarin) and esculetin (6,7-dihydroxycoumarin) were reported to exhibit an antiproliferative effect on leukemic cells by inducing apoptosis.[11,12]

Stilbenes are also a class of natural products derived from the phenylpropanoid pathway, and act as phytoalexins to defend plants against pathogen attack or environmental stress.[13] Over the course of the past decade, one of the most extensively studied stilbenes is the 3,5,4′-trihydroxy-*trans*-stilbene, known as resveratrol which is found in grapes and other food products. This molecule is endowed with several remarkable biological properties including antioxidant and cancer chemoprevention,[14–16] and its simplicity in structure has resulted in extensive effort to find more active antioxidants and cancer chemoprevention agents by synthesizing its analogs.[16,17] We recently synthesized resveratrol analogs by the introduction of electron-donating (ED) groups in the *ortho*- or *para*-position of 4-OH or 4′-OH[18,19] and elongation of the conjugated links,[20] and found that the two structural modifications are the important strategies to improve the antioxidant and antiproliferative activities of the resveratrol. For instance, a resveratrol analogue, 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), which contains two OH in 4 and 4′ positions, exhibited stronger antioxidant activity and antiproliferative activity against human promyelocytic leukemia HL-60 cells than resveratrol.[19] The two activities were further strengthened by elongation of the conjugated links as examplified in a triene compound (*trans*,*trans*,*trans*-1,6-bis(4-hydroxyphenyl)-1,3,5-hexatriene) bearing the same 4,4′-dihydroxy groups.[20] Additionally, incorporating or linking the skeletons of two or more kinds of natural products with similar biological properties by the hybrid approach, has recently attracted much attention and opened new avenues in drug discovery.[21] We have also constructed resveratrol analogs by incorporating a chroman moiety of vitamin E, leading to the remarkable increase in radical-scavenging activity.[22]

In view of these similar properties of resveratrol and coumarins, it is of interest to design and synthesize stilbene-coumarin hybrids as examplified in 3-phenylcoumarins, in which 3,4-double bond of the coumarin nucleus fixes the *trans* configuration of the stilbene double bond.[2,23–25] It has been reported that 3-phenylcoumarins can be characterized as potent and selective monoamine oxidase-B inhibitors,[23,24] vasorelaxants and platelet antiaggregants[25] as well as horseradish peroxidase catalytic activity inhibitors.[2] Encouraged by the aforementioned information, in the present work we synthesized fourteen hydroxylated 3-phenylcoumarins, **1**–**14**, including six novel *ortho*-hydroxy-methoxy substituted derivatives (**9**–**14**) with the aim of developing some compounds with antioxidant and antiproliferative activities superior to that

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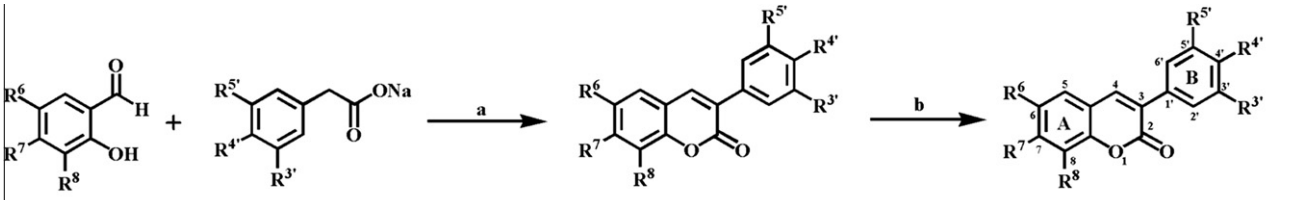
of resveratrol, and understanding the structure–activity relationship. In the compounds, different numbers and positions of hydroxy group were introduced to aromatic A and/or B ring(s). Especially, most of them contained *ortho*-hydroxy-methoxy groups and *ortho*-dihydroxy groups because the introduction of ED groups such as methoxy and hydroxy in the *ortho* position of OH, helps increase antioxidant activity.[26]

The overall strategy for the synthesis of hydroxylated 3-phenylcoumarins is outlined in Scheme 1. Methoxy and/or acetoxyl substituted 3-phenylcoumarins were prepared from the corresponding phenyl acetic acid sodium and salicylaldehyde in dry acetic anhydride by Perkin reaction.[2,27] Hydrolysis of acetoxyl group was performed with excess NH_3 solution, giving the methoxy and/or hydroxy substituted 3-phenylcoumarins, **1**–**14**. For synthetic details and characterization of all the hydroxylated 3-phenylcoumarins, as well as experimental details of the subsequent biological evaluation, please see the Supplementary data.

Free radical-mediated oxidative damage of DNA has been suggested to be a major factor in the development of cancer, thus, we first evaluated their antioxidant activity against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced plasmid pBR322 DNA strand breakage using agarose gel electrophoresis analysis (Fig. 1).[28] DNA samples were incubated with 10 mM AAPH at 37 °C for 1 h and subjected to agarose gel electrophoresis. The native DNA was mostly in its supercoiled form associated with small amount of open circular form depending on the batch (Fig. 1A and B, native lane). In the presence of AAPH, the supercoiled DNA was transformed completely into its open circular and linear forms, indicating a single and double strand breakage, respectively (Fig. 1A and B, control lane). It can be seen from Figure 1 that antioxidative effect of hydroxylated 3-phenylcoumarins against AAPH-induced DNA strand breakage depended on the specific compound used, and the majority of the compounds showed better activity than resveratrol. On the basis of the density of intact supercoiled DNA and damaged open circular and linear DNA, the activity followed the order: **6** (6,7,4′-trihydroxy groups) ≈ **7** (6,7-dihydroxy groups) ≈ **8** (6,7,3′,4′-tetrahydroxy groups) > **12** (6,7,4′-trihydroxy-3-methoxy groups) ≈ **13** (7,4′-dihydroxy-6,8-dimethoxy groups) ≈ **14** (7-hydroxy-6,8,3′,4′,5′-pentamethoxy groups) > **10** (7,4′-dihydroxy-3′-methoxy groups) ≈ **11** (7,4′-dihydroxy-6,3′-dimethoxy groups) ≈ **5** (7,3′,4′-trihydroxy groups) > **3** (7,4′-dihydroxy groups) ≈ **4** (7,3′,5′-trihydroxy groups) ≈ resveratrol > **9** (7,4′-dihydroxy-6-methoxy groups) > **1** (7-hydroxy group) > **2** (4′-hydroxy group). Among them, the compounds (**6**, **7**, and **8**) bearing *ortho*-dihydroxy groups on aromatic A ring were the most active, and completely protected plasmid DNA from strand breakage induced by AAPH. The higher activity of the compounds bearing *ortho*-dihydroxy groups is usually explained by stabilization of *ortho*-hydroxyphenoxyl radical through formation of an intramolecular hydrogen bond.[26] A comparison of the activity for **6** and **5**, indicates clearly that *ortho*-dihydroxy groups on the aromatic A ring are more active than that on the aromatic B ring. The same tendency was also observed between monohydroxy derivatives (**1** and **2**), in which hydroxy group on the A ring is more effective than that on the B ring. Additionally, compound **3** was less potent than the compounds **10**, **11**, **13**, and **14**, suggesting that the introduction of methoxy group in the *ortho*-position of hydroxy group increases significantly the activity. However, compound **9** bearing *ortho*-hydroxy-methoxy groups was an exception, and it appeared to be less active.

We next investigated their antiproliferative effects against human promyelocytic leukemia HL-60 and human lung



Methoxy and/or acetoxyl substituted 3-phenylcoumarins: **1'-14'**

Compounds	R^6	R^7	R^8	$R^{3'}$	$R^{4'}$	$R^{5'}$
1'	H	OAc	H	H	H	H
2'	H	H	H	H	OAc	H
3'	H	OAc	H	H	OAc	H
4'	H	OAc	H	OAc	H	OAc
5'	H	OAc	H	OAc	OAc	H
6'	OAc	OAc	H	H	OAc	H
7'	OAc	OAc	H	H	H	H
8'	OAc	OAc	H	OAc	OAc	H
9'	OMe	OAc	H	H	OAc	H
10'	H	OAc	H	OMe	OAc	H
11'	OMe	OAc	H	OMe	OAc	H
12'	OAc	OAc	H	OMe	OAc	H
13'	OMe	OAc	OMe	H	OAc	H
14'	OMe	OAc	OMe	OMe	OMe	OMe

Hydroxylated 3-phenylcoumarins: **1-14**

Compounds	R^6	R^7	R^8	$R^{3'}$	$R^{4'}$	$R^{5'}$
1	H	OH	H	H	H	H
2	H	H	H	H	OH	H
3	H	OH	H	H	OH	H
4	H	OH	H	OH	H	OH
5	H	OH	H	OH	OH	H
6	OH	OH	H	H	OH	H
7	OH	OH	H	H	H	H
8	OH	OH	H	OH	OH	H
9	OMe	OH	H	H	OH	H
10	H	OH	H	OMe	OH	H
11	OMe	OH	H	OMe	OH	H
12	OH	OH	H	OMe	OH	H
13	OMe	OH	OMe	H	OH	H
14	OMe	OH	OMe	OMe	OMe	OMe

Scheme 1. Synthesis of hydroxylated 3-phenylcoumarins, **1**–**14**. Reagents and conditions: (a) dry acetic anhydride, reflux, 8 h; (b) NH_3 solution (15%), ethanol, rt, 12 h.

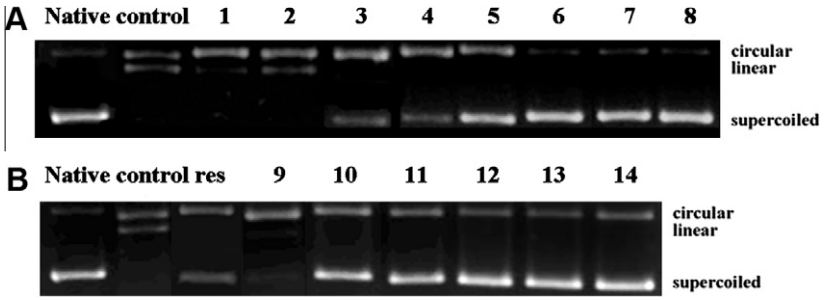


Figure 1. Protection against AAPH-induced pBR322 DNA strand breakage by hydroxylated 3-phenylcoumarins. DNA (100 ng/25 μl) was incubated with 10 mM AAPH and 20 μM hydroxylated 3-phenylcoumarins at 37 °C for 1 h in 10 mM PBS (pH 7.4). Lanes: native, only DNA; control, DNA and AAPH; others, DNA, AAPH and compounds **1**–**14** or resveratrol (res), respectively.

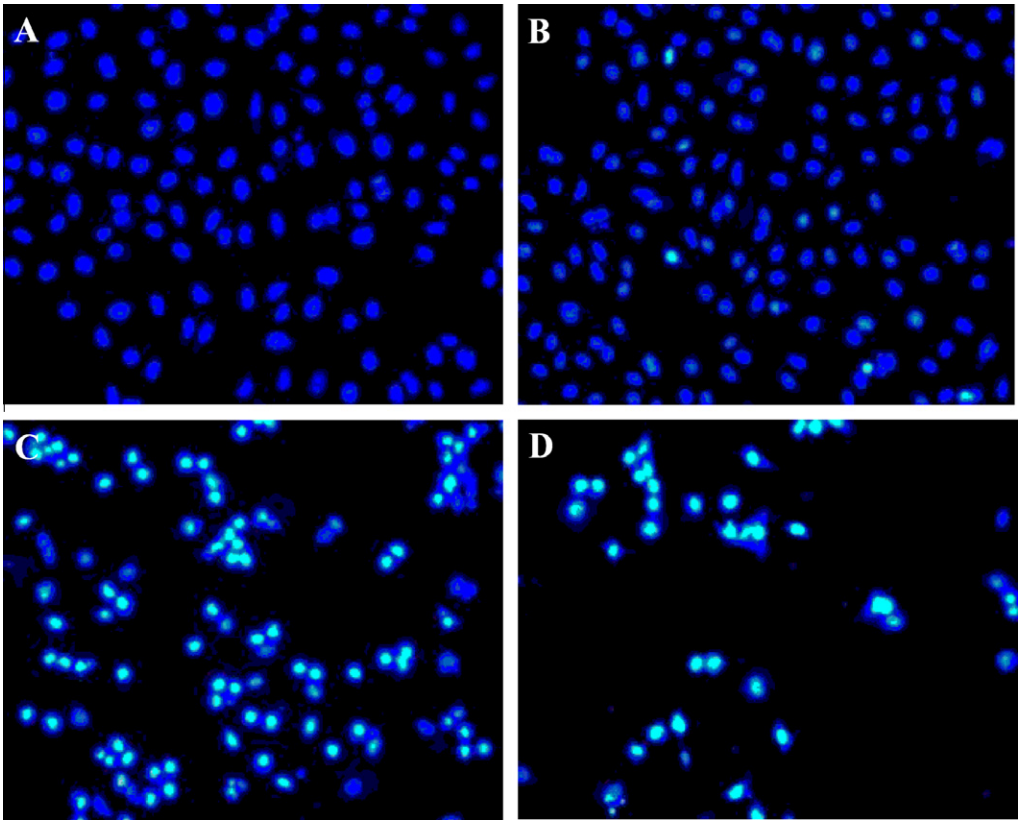


Figure 2. Effect of **9** on morphology of A549 cells. A549 cells were treated with culture medium, 5, 10, and 15 μM of **9**, respectively (A–D) for 24 h prior to analysis of cell morphology using Hoechst 33258/propidium iodide nuclear staining and fluorescence microscopy (original magnification, 200×). Living cells was blue-stained with smooth appearance; early apoptotic cells was blue-stained with bright specks of condensed chromatin. Data were representative of three separate experiments.

adenocarcinoma epithelial A549 cells by MTT assay[29] and the IC_{50} values are summarized in Table 1. Compounds **6**, **7**, **8**, **9**, and **11** exhibited higher antiproliferative activity than resveratrol on HL-60 and A549 cells. A comparison of the antiproliferative activity for compounds **5**–**8** or **9**–**11**, suggests clearly that *ortho*-dihydroxy or *ortho*-hydroxy–methoxy groups on the aromatic A ring contributes critically the activity. It can also be seen from Table 1 that the compounds (i.e., **6**–**8**) with high antioxidant activity exhibited specific antiproliferative activity, whereas the compounds (i.e., **1**–**3**) with weak antioxidant activity were inactive in the antiproliferative effects. However, interestingly, a new derivative **9** bearing *ortho*-hydroxy-methoxy groups on the aromatic A ring, behaved as a poor antioxidant but displayed the highest potency among all compounds tested with IC_{50} values of 5.2 and 7.5 μM in HL-60 and A549 cells. The compound was about 7 and 16 times more active than resveratrol in HL-60 and A549 cells, respectively. Therefore, there is not a very direct relationship between the antiproliferative activity and antioxidant ability.

Further, to determine whether the excellent antiproliferative activity of **9** is due to apoptotic cell death, A549 cells were treated with the compound (5–15 μM) for 24 h, and change in nuclear morphology was observed by fluorescence microscopy using hoechst 33258/propidium iodide staining according to the reported procedure with some modifications.[30–32] As shown in Figure 2, **9** altered significantly cell morphology with respect to those of control cultures, and caused a significant reduction in the level of living cell and a clear early apoptosis characteristic by blue-stained cells with bright specks of condensed chromatin, in a concentration-dependent manner. Finally, to explore whether **9** has a cell cycle arrest effect in A549 cells, the cells treated with different concentrations of the compound for 24 h were subjected to flow cytometric analysis after propidium iodide staining.[32] It

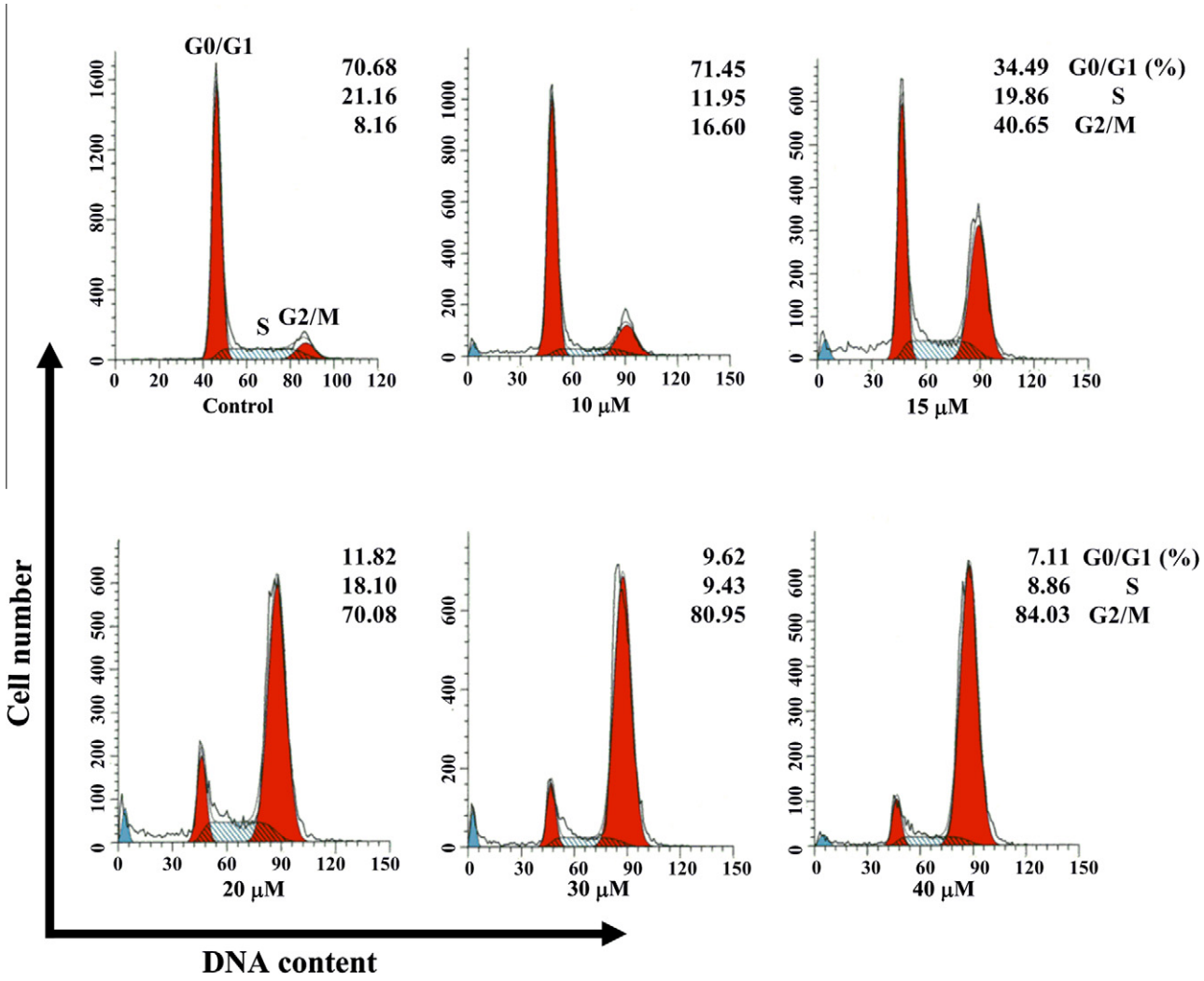
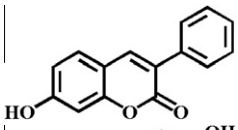
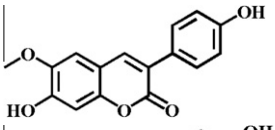
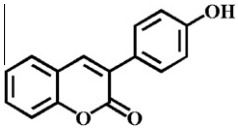
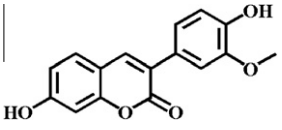
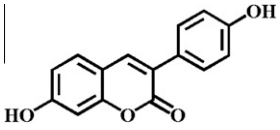
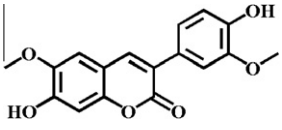


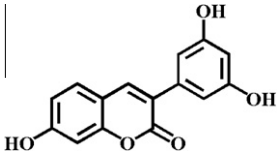
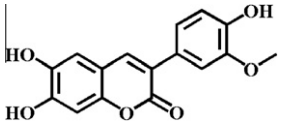
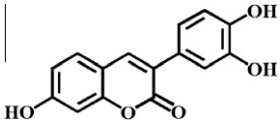
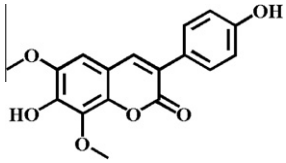
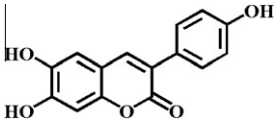
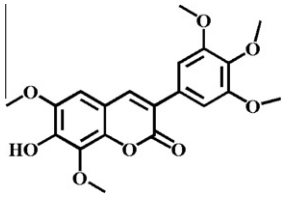
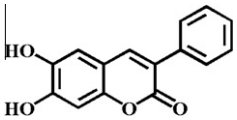
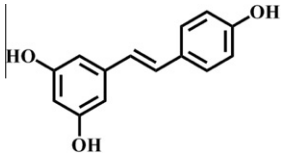
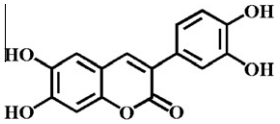
Figure 3. A549 cell cycle changes in response to treatment of **9**. After A549 cells were incubated with culture medium, 10, 15, 20, 30, and 40 μM **9** for 24 h, cell cycle distribution was determined using flow cytometry with propidium iodide staining expressed as percent of G0/G1, S, and G2/M cycle phases. Data were representative of three separate experiments.

Table 1
Antiproliferative activity of hydroxylated 3-phenylcoumarins on HL-60 and A549 cells[a]

Compounds		IC_{50} (μM)		Compounds		IC_{50} (μM)	
		HL-60	A549			HL-60	A549
1		>200	>200	**9**		5.2 ± 0.6	7.5 ± 0.6
2		>200	>200	**10**		91.7 ± 1.8	>200
3		>200	>200	**11**		24.3 ± 0.6	21.4 ± 1.8

(*continued on next page*)

Table 1 (*continued*)

Compounds		IC_{50} (μM)		Compounds		IC_{50} (μM)	
		HL-60	A549			HL-60	A549
4		127.0 ± 0.1	>200	**12**		—[b]	43.4 ± 3.9
5		42.1 ± 4.0	85.0 ± 1.6	**13**		25.1 ± 1.5	45.8 ± 3.1
6		25.0 ± 0.1	42.5 ± 2.7	**14**		72.2 ± 4.5	>200
7		14.0 ± 1.2	27.0 ± 1.3			36.3 ± 1.8[c]	119.6 ± 6.9
8		22.1 ± 2.0	48.8 ± 6.3				

[a] HL-60 and A549 cells were treated with hydroxylated 3-phenylcoumarins for 48 and 72 h, respectively. Antiproliferative activity is expressed as IC_{50} values, the concentration for the compound to cause 50% inhibition of cell proliferation. Data are expressed as the mean ± SD for three determinations.
[b] The accurate IC_{50} values could not be measured because of the formation of other color compounds in the experiments.
[c] Cited from Ref. 19.

can be seen from Figure 3 that **9**-treated cells were remarkably blocked in the G2/M phase depending on its doses, and this change in G2/M phase was accompanied by a decrease in the proportion of cells in G0/G1 phase. Specifically, **9** increased the percentage of cells in the G2/M phase to 16.6, 40.65, 70.08, 80.95 and 84.03% at 10, 15, 20, 30, and 40 μM, respectively. The above two results suggest that the excellent antiproliferative activity of **9** could be mediated by deregulation in cell cycle and induction of apoptosis.

In summary, our study shows that most of hydroxylated 3-phenylcoumarins (stilbene-coumarin hybrids) are effective antioxidants against AAPH-induced pBR322 DNA strand breakage. Moreover, the hybrids with *ortho*-dihydroxy groups or *ortho*-hydroxy-methoxy groups on the aromatic A ring exhibit superior antiproliferative activity in comparison with those with such groups on the aromatic B ring. Specially, a new hybrid bearing *ortho*-hydroxy–methoxy groups on the aromatic A ring, **9** emerged as an important lead compound with excellent antiproliferative, apoptosis-inducing and cell cycle arrest activities.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.090.

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