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Synthesis, growth inhibition, and cell cycle evaluations of novel flavonoid derivatives

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Abstract—As a continuation of our search for potential new anticancer agents, a series of ten flavonoid derivatives has been synthesized by cyclization of substituted chalcones. Target compounds were evaluated for their biological activity. Among them, compounds **1**–**4** and **9** displayed a significant growth inhibitory action against a panel of tumor cell lines including Jurkat, PC-3, and Colon 205. On treatment with an equitoxic (IC_{50}) concentration, compounds **1**–**5** and **7**–**9** blocked cells in the G2/M phase of the Jurkat cell cycle, whereas compound **6** blocked the same in the G0/G1 phase.
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1. Introduction

Many clinically successful anticancer drugs were themselves either naturally occurring molecules or have been developed from their synthetic analogs. Great interest is currently being paid to natural products for their interesting anticancer activities. Flavonoids are prominent plant secondary metabolites that are consumed by humans as dietary constituents in amounts exceeding 0.1 g/day. A large number of biological activities have been attributed to these compounds including anticancer.[1] The interaction of dietary flavonoids with the gut has numerous implications for human health, and flavonoid compounds in the diet may act as chemopreventive agents against the development of cancer in the alimentary tract.[2] In fact, a number of studies have demonstrated anticancer activity associated with flavonoids;[3] for example, flavone has been shown to be a potent apoptosis inducer in human colon carcinoma cells, and this effect appears to be restricted to cancer cells.[4] Similarly, selective induction of apoptosis in human prostate cancer cells by apigenin[5] and in human myeloid leukemia HL-60 cells by luteolin has been reported recently.[6] Induction of apoptosis by wogonin and fisetin has been demonstrated to involve activation of the caspase 3 cascade in hepatocellular carcinoma SK-HEP-1 cells and human leukemia HL-60 cells.[7,8] Further, two prospective cohort studies in Finland, where average flavonoid intakes are relatively low, found that men with the highest dietary intakes of flavonols and flavones had a significantly lower risk of developing lung cancer than those with the lowest intakes.[9] More recently, flavone-8-acetic acid derivatives have been reported as reversible inhibitors of aminopeptidase N/CD13.[10] Importantly, a flavonoid derivative flavopiridol was widely used in traditional medicine and was a novel semisynthetic flavone analog of rohitukine, a leading anticancer compound derived from an Indian tree. Flavopiridol has been found to inhibit cyclin-dependent kinases (Cdks), induce apoptosis, suppress inflammation, and modulate the immune response,[11] and was the first compound with this activity to have entered the clinic as an anticancer drug.

As part of our continuing search for potential anticancer drug candidates, we have recently described the growth inhibitory properties and cell cycle analysis of novel flavonoid (chalcone) derivatives using a panel of cancer cell lines.[12] Based on the diverse biological activities of flavonoid derivatives, in the present study we have synthesized a series of natural flavonoids and examined their in vitro cytotoxic activity on human tumor cell lines, namely Jurkat (human lymphocytic cancer cell line), PC-3 (human prostate cancer cell line), HepG2 (human hepatoma cancer cell line), Colon 205 (human colonic cancer cell line), and the normal peripheral blood mononuclear cells (PBMCs). In addition, to

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obtain some preliminary insights into the mechanism (s) of action, these compounds' effects on Jurkat cell cycle analysis were also examined.

2. Results and discussion

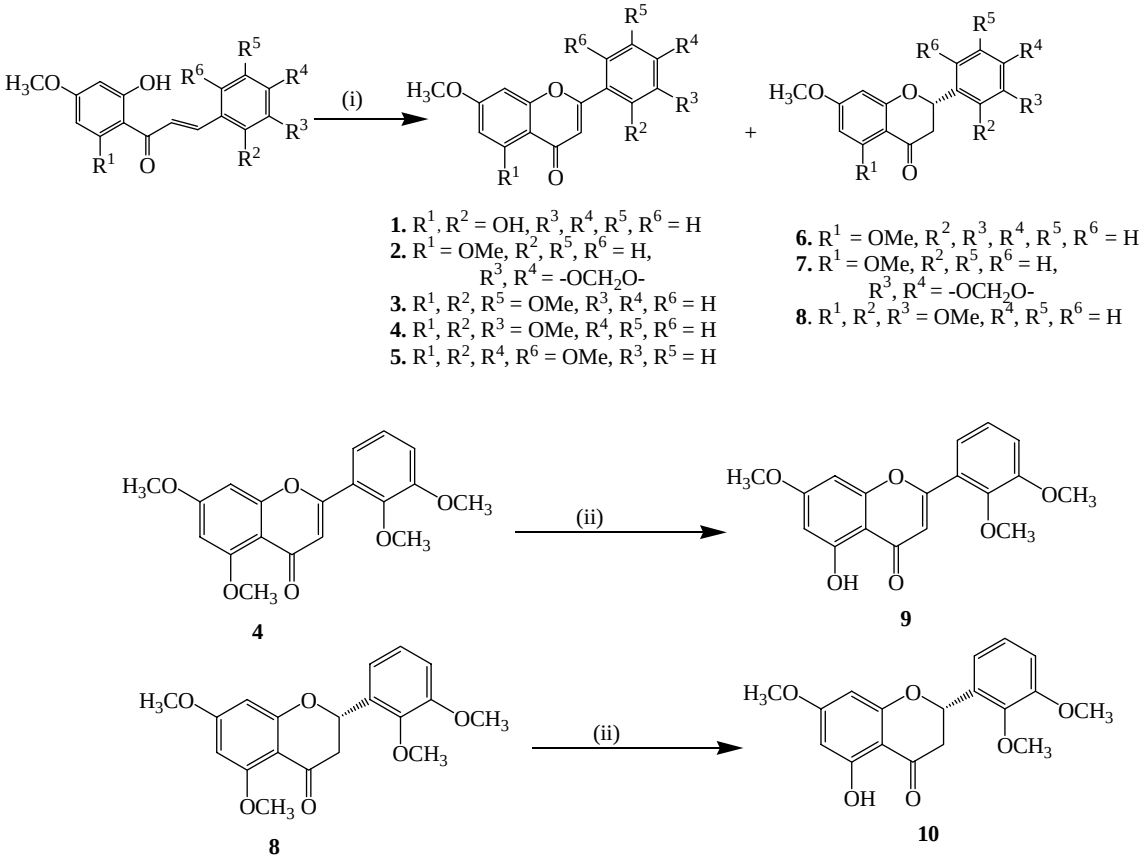
2.1. Chemistry

Compounds described in this study were prepared following a straightforward chemistry, and are shown in Figure 1. The starting compounds (chalcones) were synthesized by Claisen–Schmidt condensation between substituted 2′-hydroxyacetophenones and benzaldehydes in alkaline medium, according to our previously described method.[12] Studies with the model compound **4** on cyclization of these chalcones revealed that the reaction was affected by temperature. When these chalcones were oxidized with 2 equiv of DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) in dioxane at 110 °C, a single compound, flavone, was generated in 90% yield, as reported previously.[13–15] In fact, our aim was to highlight the possibility of obtaining several products by gradual variation of reaction conditions. Therefore, when the temperature-controlled at 60 °C, the chalcones were cyclized with DDQ in dioxane to the corresponding flavones and flavanones. Despite modest yield, this procedure involves mild conditions, a one-step reaction, does not need any protection of hydroxyl groups, and affords a range of natural flavones and flavanones. 5-Hydroxy 7,2′,3′-trimethoxyflavone (**9**) and 5-hydroxy 7,2′,3′-trimethoxyflavanone (**10**) were prepared from the corresponding 5-methoxy compounds **4** and **8** by demethylation with $AlCl_3$ in CH_3CN because this method can selectively cleave the methyl ether β to the carbonyl group.[16] However, we have not studied further various products or relative yields from demethylation reactions.

The majority of flavonoids as shown in Figure 1, occur in nature confirming the versatility of our method for the synthesis of natural flavonoids. Compounds **1**, **3**–**5**, and **8**–**10** were natural products found in *Andrographis* spp.[17–19] Compound **2** was reported from the fruits of *Neoraputia magnifica*.[20] Flavonoids **6** and **7** were other natural compounds and can be isolated from *Caesalpinia pulcherrima*.[21] All the synthesized flavonoid derivatives were chemically characterized by melting point (mp), infrared (IR) and nuclear magnetic resonance (^{1}H NMR) spectra, as well as mass spectra (MS).

2.2. Cytotoxicity

The newly synthesized flavonoid derivatives were examined for their cytotoxic properties in a panel of four human tumor cell lines containing examples of lymphocytic (Jurkat), prostate (PC-3), hepatoma (HepG2), and colonic (Colon 205). To determine the degree of toxicity of these compounds toward healthy cells, experiments were also carried out under the same



1. R^1, R^2 = OH, R^3, R^4, R^5, R^6 = H
2. R^1 = OMe, R^2, R^5, R^6 = H, R^3, R^4 = -OCH_2O-
3. R^1, R^2, R^5 = OMe, R^3, R^4, R^6 = H
4. R^1, R^2, R^3 = OMe, R^4, R^5, R^6 = H
5. R^1, R^2, R^4, R^6 = OMe, R^3, R^5 = H

6. R^1 = OMe, R^2, R^3, R^4, R^5, R^6 = H
7. R^1 = OMe, R^2, R^5, R^6 = H, R^3, R^4 = -OCH_2O-
8. R^1, R^2, R^3 = OMe, R^4, R^5, R^6 = H

Figure 1. Reagents and conditions: (i) DDQ, 1,4-dioxane, 60 °C, 6 h, (ii) $AlCl_3$, CH_3CN, reflux, 1 h.

experimental conditions in normal PBMCs. All the results presented were obtained by two independent methods: cell density measurement—Trypan blue exclusion method and cellular viability assessment—MTT colorimetric assay. A treatment period of 3 days was selected since the control cells were still in the exponential growth phase at this time. The IC_{50} (drug concentration required to reduce the initial cell number by 50%) values, calculated from the dose-survival curves obtained after 72 h of compound treatment from MTT test, are shown in Table 1. These results showed that flavones **1**–**4** and **9** exhibited significant cytotoxic activity, with IC_{50} values below 100 μM, against all of the cell lines, except the hepatoma cancer cell line HepG2, confirming the importance of the C_2–C_3 double bond for flavonoid cytotoxicity.[22] A comparison of the potencies of **4** and **9** suggests that substitution of the hydroxyl with a methoxy group reduces the activity; in addition, **4** possesses the hydrogen bond of the 5-hydroxyl group with the 4-ketonic oxygen and was therefore more hydrophobic than **9**.[22] On the other hand, the flavanones **6**–**8** and **10** had negligible cytotoxic effects against all cell lines tested, as their IC_{50} values were generally higher than 100 μM, which suggested that these compounds displayed growth inhibitory activities to human tumor cells and caused neither normal nor cancer cell death, even when the concentrations of these compounds were as high as 100 μM. This finding may be useful to understand the major drawback in the application of anticancer drugs to clinical therapies, bringing a host of side effects due to a large number of normal functional cells being destroyed by the increasing dosages of antitumor agents. This phenomenon was unusual and meaningful to put forward medical therapies on human cancer diseases, whereas drugs with higher cytotoxic activities always inflicted a the higher damage to normal cells. Further, the data in this study indicate that the tumor cell lines were more sensitive to the tested compounds, while normal cells (PBMCs) are relatively resistant to these drugs. In the presence of these compounds, cell growth of the human cancer cell lines Jurkat, PC-3, and Colon 205 was inhibited, which was indicated by a range of low IC_{50} values, whereas the cell growth in the normal PBMCs was less inhibited as shown by higher IC_{50} values (Table 1).

2.3. Cell cycle analysis

The eukaryotic cell cycle was a series of tightly regulated events that result in the transmission of genetic material from one generation to the next. Fidelity of these processes was monitored by cell cycle checkpoints that induce cell cycle arrest whenever any damage was detected. If the damage cannot be rectified, these checkpoints may also provoke cells to undergo apoptosis. Many chemotherapeutic agents take advantage of these checkpoint controls to preferentially kill rapidly dividing cancer cells. However, there were several problems common to the existing cancer chemotherapies which mainly result from a narrow therapeutic index leading to any undesired side effects. Increased drug resistance in tumors was another problem.[23] Thus, there is still need for effective drugs with an improved safety profile.

Parallel to the cytotoxic studies described above, a cell-cycle analysis was performed for compounds **1–10** to determine whether these flavonoid derivatives influence the progression of cell cycle of the Jurkat cell line. As shown in Table 2, Jurkat cells were exposed to the vehicle solvent (DMSO) as control, and IC_{50} concentrations of compounds **1**–**10** were added to the cell line. After exposure of the compounds for 24 h, attached cells were

Table 2. Cell cycle perturbations induced by flavonoid derivatives **1**–**10**[a]

Compound	G0/G1	S	G2/M
Control	33.17	82.62	1.21
1	34.15*	0.25***	65.6***
2	35.67*	2.01***	62.32***
3	32.32*	9.76***	57.92***
4	28.89*	26.87**	44.24***
5	29.19*	2.47***	68.34***
6	66.07***	31.6**	2.33
7	32.32*	9.76***	57.92***
8	32.37*	0.5***	67.13***
9	41.02**	8.09***	50.89***
10	29.28*	68.55*	2.16

*,**, and ***Represents $p < 0.05$, 0.01, and 0.001, respectively, compared to control.

[a] Cells were treated with an IC_{50} value of each compound for 24 h and then the cell cycle was analyzed by flow cytometry analysis.

Table 1. Cell growth inhibition in the presence of newly synthesized flavonoid derivatives

Compound	IC_{50} (μM) ± SD				
	Jurkat	PC-3	HepG2	Colon 205	PBMCs
1	20	50	200	45	150
2	15	20	>200	25	>200
3	35	45	>200	90 (75%)[a]	>200
4	25	50	200 (85%)[a]	50	200 (85%)[a]
5	60	100 (85%)[a]	>200	100 (80%)[a]	150
6	100	>200	>200	>200	>200
7	100	>200	>200	>200	>200
8	100	200 (85%)[a]	>200	200 (85%)[a]	>200
9	15	30	200 (85%)[a]	30	>200
10	100	>200	>200	200 (80%)[a]	>200

[a] Percentage of inhibition when compared with control values.

analyzed by flow cytometry. The majority of control cells exposed to DMSO were either in the G0/G1 phase (33.17%) or the S phase (82.62%) of the cell cycle and only a few cells in the G2/M phase were detected (1.21%) (Table 2). After treatment with compounds **1**–**10** for 24 h, an accumulation of G2/M stage cells in the **1**–**5** and **7**–**9** samples was observed. The percentage of cells in the G2/M phase increased in a range from 1.21% to 68.34% and 67.13% after treatment of Jurkat cells with **5** and **8**. A similar G2/M blockage was also induced by compounds **1**–**4**, **7**, and **9**. It was assumed that G2/M phase arrest was possible due to the inhibition of enzymes essential for G2/M progression or mitosis.[24] In contrast, compound **6** increased the proportion of cells in the G0/G1 phase, while it decreased the percentage of those in the S phase, the cells found in the G0/G1 phase was 66.07%, compared to 33.17% for untreated cells after 24 h of treatment. This finding indicates either (a) an inhibition of progression through the G1 phase or an inhibition of transition from G1 into the S phase or (b) that the cells exited from the cell cycle and entered the G0 phase.[25] Unfortunately, none of the flavonoid derivatives showed any significant S-phase arrest in Jurkat cells at their IC_{50} concentration, and only compound **10** had significant effects on cell cycle progression. To the best of our knowledge, this is the first report of the compounds **1**–**10** for their cell cycle analysis on the Jurkat cell line.

In summary, the present work describes the first report on the synthesis of natural flavonoid derivatives (**1**–**10**) and evaluation of their cytotoxic activities. Representative compounds inhibited the in vitro growth of various human cancer cells including lymphocytic, colon, and prostate. These results revealed that the alkenone moiety plays an important role in cytotoxicity. Further, this study also revealed several specific G2/M phase blocker lead compounds. A more detailed biological evaluation of these and related compounds will be reported in due course.

3. Materials and methods

3.1. Chemistry

Unless otherwise noted, chemicals were commercially available and used as received without further purification. Melting points were determined with a Buchi-510 melting point apparatus and are not corrected. Infrared spectra were recorded on KBr disks on a Perkin-Elmer FT-IR paragon 500 spectrometer. UV spectra were obtained in MeOH on a Varian Cary Win UV-50 spectrophotometer. ESI-MS spectra were recorded on a Thermo-Finnigan LCQ Advantage system. ^{1}H NMR spectra were measured on a Varian Unity Inova-600 VXR-300/51 spectrometer, using the indicated solvents; chemical shifts were reported in δ (ppm) downfield from TMS as an internal reference. Coupling constants are given in Hertz. In case of multiplets, the chemical shift quoted was measured from an approximate center. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Silica gel for column chromatography (CC) (0.063–0.200 mm), was a product of Merck. TLC was performed on Merck TLC plates (0.23 mm thickness), with compounds visualized by spraying with 8% (v/v) H_2SO_4 in EtOH and then heating on a hot plate.

3.2. General procedure for the synthesis of flavonoid derivatives (1–8)[15]

Substituted chalcone (0.01 mol) was dissolved in 1,4-dioxane (10 mL). To the solution was added DDQ (0.01 mol). The ensuing mixture was stirred for 6 h at 60 °C. The solvent was removed in vacuo. Water (50 mL) was added and the mixture was extracted with EtOAc (3 × 50 mL), dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography over silica gel, eluting with gradient of *n*-hexane/EtOAc to yield the corresponding substituted flavonoids (**1**–**8**) (Fig. 1).

3.2.1. 5,2′-Dihydroxy 7-methoxyflavone (1). This compound was prepared by the general method of **2** with 2,2′,6′-trihydroxy 4′-methoxychalcone as a starting material, yield 1.68 g, 58.7%, white solid; mp 258–260 °C; UV (MeOH) λ_{max} (log ε): 262 (4.32), 330 (4.10) nm; IR (KBr) ν_{max}: 3350, 2990, 1660, 1615, 1520; ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.80 (OH-5), 10.82 (OH-2′), 7.88 (1H, dd, J = 7.8, 3.0 Hz, H-6′), 7.35 (1H, dt, J = 7.8 Hz, H-4′), 7.15 (1H, s, H-3), 6.95-7.02 (2H, m, H-3′, 5′), 6.70 (1H, d, J = 3.0 Hz, H-8), 6.31 (1H, d, J = 3.0 Hz, H-6), 3.87 (3H, s, OMe-7); ESI-MS (positive mode) m/z 308.1 $[M+Na]^+$.

3.2.2. 5,7-Dimethoxy 3′,4′-methylenedioxyflavone (2). This compound was prepared by the general method of **2** with 2′-hydroxy 3,4-methylenedioxy 4′,6′-dimethoxychalcone as a starting material, yield 1.71 g, 52.1%, white solid, mp 160–162 °C; UV (MeOH) λ_{max} (log ε): 260 (4.12), 325 (3.96) nm; IR (KBr) ν_{max}: 1655, 1610, 1495; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.45 (1H, d, J = 3.0 Hz, H-2′), 7.41 (1H, dd, J = 8.0, 3.0 Hz, H-6′), 7.15 (1H, s, H-3), 6.96 (1H, d, J = 8.0 Hz, H-5′), 6.82 (1H, d, J = 2.5, H-8), 6.40 (1H, d, J = 2.5 Hz, H-6), 6.14 (2H, s, -OCH_2O-), 3.89 (3H, s, OMe-7), 3.81 (3H, s, OMe-5); ESI-MS (positive mode) m/z 350.1 $[M+Na]^+$.

3.2.3. 7,2′,5′-Trimethoxyflavone (3). This compound was prepared by the general method of **2** with 2′-hydroxy 2,4′,5-trimethoxychalcone as a starting material, yield 1.90 g, 60.5%, yellow amorphous powder; mp 136–138 °C; UV (MeOH) λ_{max} (log ε): 255 (4.24), 330 (4.13) nm; IR (KBr) ν_{max}: 2940, 1645, 1618, 1584; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.13 (1H, d, J = 9.0 Hz, H-5), 7.44 (1H, d, J = 3.6 Hz, H-6′), 7.11 (1H, s, H-3), 7.03 (1H, dd, J = 9.0, 3.6 Hz, H-4′), 6.98 (2H, m, H-3′, 8), 6.92 (1H, d, J = 2.4 Hz, H-6), 3.92 (3H, s, OMe-7), 3.89 (3H, s, OMe-2′), 3.86 (3H, s, OMe-5′); ESI-MS (positive mode) m/z 313.3 $[M+H]^+$.

3.2.4. 5,7,2′,3′-Tetramethoxyflavone (4). This compound was prepared by the general method of **2** with 2′-hydroxy 2,3,4′,6′-tetramethoxychalcone as a starting material, yield 1.86 g, 54.0%, yellow crystals, mp 150–152 °C;

UV (MeOH) λ_{max} (log ε): 260 (3.98), 310 (3.76) nm; IR (KBr) ν_{max}: 2930, 1656, 1612, 1575; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.32 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.17 (1H, t, J = 7.8 Hz, H-5′), 7.05 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 6.83 (1H, s, H-3), 6.51 (1H, d, J = 3.0 Hz, H-8), 6.38 (1H, d, J = 3.0 Hz, H-6), 3.96 (3H, s, OMe-5), 3.92 (3H, s, OMe-3′), 3.90 (3H, s, OMe-7), 3.88 (3H, s, OMe-2′); ESI-MS (positive mode) m/z 366.1 $[M+Na]^+$.

3.2.5. 5,7,2′,4′,6′-Pentamethoxyflavone (5). This compound was prepared by the general method of **2** with 2′-hydroxy 2,4,4′,6,6′-pentamethoxychalcone as a starting material, yield 2.01 g, 53.7%, pale yellow solid, mp 192–194 °C; UV (MeOH) λ_{max} (log ε): 256 (4.38), 304 (4.18) nm; IR (KBr) ν_{max}: 2940, 1660, 1612, 1585; ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.44 (1H, d, J = 2.4 Hz, H-8), 6.33 (1H, d, J = 2.4 Hz, H-6), 6.22 (1H, s, H-3), 6.14 (2H, s, H-3′, 5′), 3.93 (3H, s, OMe-5), 3.84 (3H, s, OMe-4′), 3.83 (3H, s, OMe-7), 3.75 (6H, s, OMe-2′, 6′); ESI-MS (positive mode) m/z 395.1 $[M+Na]^+$.

3.2.6. 5,7-Dimethoxyflavanone (6). This compound was prepared by the general method of **2** with 2′-hydroxy 4′,6′-dimethoxychalcone as a starting material, yield 1.2 g, 42.2%, white needles, mp 98–100 °C; UV (MeOH) λ_{max} (log ε): 282 (4.20), 325 sh (3.85) nm; IR (KBr) ν_{max}: 3140, 1645, 1600, 1505 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.47 (2H, dd, J = 9.0, 1.8 Hz, H-2′, 6′), 7.42 (2H, dt, J = 9.0, 1.8 Hz, H-3′, 5′), 7.37 (1H, m, H-4′), 6.17 (1H, d, J = 2.4 Hz, H-8), 6.10 (1H, d, J = 2.4 Hz, H-6), 5.41 (1H, dd, J = 13.2, 3.0 Hz, H-2), 3.90 (3H, s, OMe-7), 3.82 (3H, s, OMe-8), 3.02 (1H, dd, J = 16.2, 13.2 Hz, H-3_{ax}), 2.80 (1H, dd, J = 16.2, 3.0 Hz, H-3_{eq}); ESI-MS (positive mode) m/z 285.1 $[M+H]^+$.

3.2.7. 5,7-Dimethoxy 3′,4′-methylenedioxyflavanone (7). This compound was obtained together with compound **2** in the same reaction as colorless needles, yield 0.90 g, 27.4%; IR (KBr) ν_{max}: 3090, 1665, 1620, 1575 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.05 (1H, d, J = 1.8 Hz, H-2′), 6.96 (1H, dd, J = 8.4, 1.8 Hz, H-6′), 6.86 (1 H, d, J = 8.4 Hz, H-5′), 6.20 (1H, d, J = 2.4 Hz, H-8), 6.13 (1H, d, J = 2.4 Hz, H-6), 6.10 (2H, s, -OCH_2O-), 5.39 (1H, dd, J = 13.2, 3.0 Hz, H-2), 3.91 (3H, s, OMe-7), 3.84 (3H, s, OMe-5), 3.03 (1H, dd, J = 15.6, 13.2 Hz, H-3_{ax}), 2.78 (1H, dd, J = 15.6, 3.0 Hz, H-3_{eq}); ESI-MS (positive mode) m/z 329.1 $[M+H]^+$.

3.2.8. 5,7,2′,3′-Tetramethoxyflavanone (8). This compound was obtained together with compound **4** in the same reaction as colorless needles, yield 1.21 g, 35.1%; mp 164–166 °C; UV (MeOH) λ_{max} (log ε): 264 (4.20), 335 sh (3.85) nm; IR (KBr) ν_{max}: 1665, 1610, 1595 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.13 (2H, m, H-5′, 6′), 6.92 (1H, dd, J = 7.8, 1.8 Hz, H-4′), 6.12 (1H, d, J = 2.4 Hz, H-8), 6.08 (1H, d, J = 2.4 Hz, H-6), 5.75 (1H, dd, J = 13.2, 3.0 Hz, H-2), 3.89 (3H, s, OMe-5′), 3.87 (3H, s, OMe-5), 3.84 (3H, s, OMe-3′), 3.80 (3H, s, OMe-7), 2.96 (1H, dd, J = 16.2, 13.2 Hz, H-3_{ax}), 2.76 (1H, dd, J = 16.2, 3.0 Hz, H-3_{eq}); ESI-MS (positive mode) m/z 368.1 $[M+Na]^+$.

3.3. General procedure for the preparation of compounds 9 and 10[16]

A mixture of substituted 5-methoxyflavonoid derivative (0.1 mmol) and anhydrous aluminium chloride (0.4 mmol) in acetonitrile (5 mL) was refluxed for 1 h. After standing at room temperature for 2 h, the mixture was poured into ice water and then neutralized with dilute HCl. The aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with a saturated NaCl, dried, filtered, and concentrated. The residue was purified by column chromatography over silica gel, eluting with a gradient of *n*-hexane/EtOAc to yield the corresponding substituted flavonoids **9** and **10** (Fig. 1).

3.3.1. 5-Hydroxy 7,2′,3′-trimethoxyflavone (9). This compound was prepared by the general method of **3** with 5,7,2′,3′-tetramethoxyflavone (**4**) as a starting material, yield 0.30 g, 88.7%, yellow amorphous solid, mp 190–192 °C; UV (MeOH) λ_{max} (log ε): 245 (3.68), 330 (3.00) nm; IR (KBr) ν_{max}: 3160, 2920, 1656, 1610; 1H NMR (600 MHz, $CDCl_3$): δ 12.77 (1H, s, OH-5), 7.32 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.17 (1H, t, J = 7.8 Hz, H-5′), 7.06 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 6.86 (1H, s, H-3), 6.43 (1H, d, J = 2.4 Hz, H-8), 6.36 (1H, d, J = 2.4 Hz, H-6), 3.91 (3H, s, OMe-2′), 3.87 (3H, s, OMe-7), 3.85 (3H, s, OMe-3′); ESI-MS (positive mode) m/z 352.1 $[M+Na]^+$.

3.3.2. 5-Hydroxy 7,2′,3′-trimethoxyflavanone (10). This compound was prepared by the general method of **3** with 5,7,2′,3′-tetramethoxyflavanone (**8**) as a starting material, yield 0.31 g, 90.1%, colorless solid, mp 148–150 °C; IR (KBr) ν_{max}: 3390, 2910, 1655, 1628, 1585 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.07 (1H, s, OH-5), 7.13 (2H, d, J = 9.0 Hz, H-4′, 6′), 6.92 (1H, t, J = 9.0 Hz, H-5′), 6.06 (1H, d, J = 2.4 Hz, H-8), 6.03 (1H, d, J = 2.4 Hz, H-6), 5.74 (1H, dd, J = 13.2, 3.0 Hz, H-2), 3.87 (3H, s, OMe-2′), 3.86 (3H, s, OMe-7), 3.79 (3H, s, OMe-3′), 3.03 (1H, dd, J = 16.2, 13.2 Hz, H-3_{ax}), 2.79 (1H, dd, J = 16.2, 3.0 Hz, H-3_{eq}); ESI-MS (positive mode) m/z 329.1 $[M+H]^+$.

3.4. Biological data

3.4.1. Chemicals. Compounds were dissolved in dimethylsulfoxide just before the experiments; calculated amounts of drug solution were added to the growth medium containing cells to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and L-glutamine were obtained from Sigma Chemical Co., St. Louis, USA.

3.4.2. Cell culture. Jurkat, PC-3, Colon 205, and HepG2 cell lines were obtained from ATCC (Rockville, MD). Blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque (Pharmacia) density gradient centrifugation.[26] The cell numbers were determined with a hemocytometer, and viabilities were assessed by Trypan blue dye exclusion. Cell lines were maintained in

logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using the RPMI-1640 medium containing 10% fetal calf serum (Biochrom-Seromed GmbH&Co., Berlin, Germany) and supplemented with 25 mM Hepes buffer, L-glutamine, and with antibiotics penicillin (50 U/mL) and streptomycin (50 U/mL).

3.4.3. Cytotoxic assay. The growth inhibitory effect on tumor, as well as normal cell lines were evaluated by means of MTT (tetrazolium salt reduction) assay.[27] Briefly, between 4 and 6×10^{-3} cells, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentrations (0–200 μM). Quadruplicate cultures were established for each treatment. Forty-eight hours later, each well was treated with 10 μL of a 5 mg/mL MTT saline solution, and after 5 h of incubation, 100 μL of acid-isopropanol was added. After an overnight incubation, the inhibition of cell growth induced by the tested compounds was detected by measuring the optical density (OD) with a microplate reader (Bio-Rad, model 3550, USA) at 570 nm ($OD_{570-620}$). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC_{50} values represent drug concentrations that reduced the mean absorbance at 570 nm of 50% of those in the untreated control wells.

3.4.4. Cell cycle analysis. Cells were treated with test compounds **1**–**10** for 24 h and then harvested by trypsinization. After centrifugation, cells were fixed in 70% ethanol at 4 °C and then resuspended in 20 μg/mL propidium iodide in 0.1% Triton X-100 and 0.1 mM EDTA for 15 min before analysis by a flow cytometry.[28] Flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA) with an excitation at 488 nm and an emission at 630 nm. The percentage of cell cycle distribution in the G0/G1, S, and G2/M phases was determined using the MODFIT software (Becton–Dickinson).

3.5. Statistics

All experimental data are shown as means ± SD and were accompanied by a number of experiments. For in vitro data, statistical analysis was performed using a one-way ANOVA, followed by Dunnetts post hoc test, and the significant difference was set at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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