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Differential effects of synthesized 2′-oxygenated chalcone derivatives: modulation of human cell cycle phase distribution

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Abstract—Ten structurally related 2′-oxygenated chalcone derivatives, bearing either hydroxy and/or methoxy substituents on the A and B rings, were synthesized through Claisen–Schmidt condensation. The synthesis procedure was relatively easy and had an acceptable yield. The in vitro cytotoxicities of these compounds against the human tumor cells such as Jurkat, U937 cells, and normal cells PHA stimulated PBMCs were investigated. Among those, compounds **1** ($IC_{50} = 2.5\,\mu M$), **2** (1.7 μM), and **8** (3.2 μM) showed potent inhibitory activity toward Jurkat cell line. In parallel, compounds **1** (6.7 μM), **2** (1.5 μM), and **10** (5.3 μM) showed the highest activity against U937 cell line. However, the chalcones also inhibit the PHA stimulated PBMCs cells, but the IC_{50} values were relatively high when compared to the tumor cell line values. Studies were also on the effect of synthesized chalcones on the cell cycle phase distribution. In Jurkat cell line, compounds **7** and **9** showed the highest activity and the most striking effect in reduction of the percentage of cells in the S phase, which was associated with an increase of cells in G2/M phase. In U937 cell line, compound **3** increased the proportion of cells in the G0/G1 phase and reduced the proportion in S phase. In contrast, compounds **1**, **9**, and **10** showed a decrease effect on the percentage of cells in S phase and an increase effect on the percentage of cells in the G2/M phase of the cell cycle. Whereas in the case of PHA stimulated PBMCs, compounds **1**, **4**, **8**, and **10** increased the percentage of cells in G2/M phase, which was associated with a decrease effect in the S phase of the cell cycle.

1. Introduction

Cancer is one of the leading causes of death in the present society. A great deal of efforts have been underway to treat various forms of cancer for decades; and until recently, chemoprevention of cancer is receiving its due share of attention. Among the small molecule drugs developed to prevent or treat cancer, there is a potentially important group of structurally effective features, which contain the alkenone moiety as the integral part of the molecule. Alkenones, acyclic, or cyclic, are a class of organic molecules bearing a ketone functionality and an unsaturated group (double bond). These two functional groups are in conjugated arrangement among the naturally occurring or synthetic anticancer agents.

Chalcones are a class of anticancer agents that have shown promising therapeutic efficacy for the management of human cancers. Chalcones, considered as the precursor of flavonoids and isoflavonoids, are abundant in edible plants. Chemically they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. Recent studies revealed that these chalcones had shown a wide variety of anticancer,[1–7] anti-inflammatory,[8–10] anti-invasive,[11] antituberculosis,[12] and antifungal[13] activities. Previous studies showed that the 2′-oxygenated chalcone derivatives (2POCDs) possess a wide spectrum of activities like inhibition of MCF-7 human breast cancer cells growth,[14] prostaglandin E2 production inhibition in rat peritoneal macrophages,[15] inhibition of the iNOS protein expression, and inhibition of cyclooxygenase-2 in RAW 264.7 cells.[16] In a recent article, 2′-hydroxychalcones are reported as inhibitors of peroxynitrite-mediated oxidation of low-density lipoproteins (LDL).[17] Furthermore, some 2′-aminochalcone derivatives were also found to show antitumor activity.[18] However, there is not yet specific report on the cytotoxic effects of 2POCDs on human tumor cells such as Jurkat, U937,

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and normal cells PHA stimulated primary peripheral blood mononuclear cells (PBMCs); and exploring the relevance for a potential clinical application. The present article is focused on synthesis of specific chalcones and interactions of the synthesized 2POCDs with human lymphocytic, monocytic cell lines and PHA stimulated PBMCs to address additional important aspects of chalcone pharmacology and toxicology.

2. Results

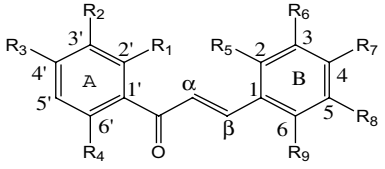
2.1. Preparation and characterization of compounds 2POCDs

The general synthetic strategy employed to prepare the chalcone derivatives was based on Claisen–Schmidt condensation, which was reported previously.[19,20] As shown in Table 1, a series of 10 chalcone derivatives (**1**–**10**), bearing either hydroxy and/or methoxy substituents on the A and B rings were prepared by base catalyzed condensation of appropriately substituted acetophenone with substituted benzaldehyde in EtOH (Scheme 1). To a stirred mixture of substituted acetophenone and substituted benzaldehyde in alcohol at 0 °C was added a 60% solution of KOH dropwise in 60 min. The reaction mixture was kept at room temperature for 2 days, then diluted with water and neutralized with 2 N HCl, then extracted with EtOAc. After purification of the residue obtained, chalcones were obtained yields of 45–88%. The structures of all the 10 chalcones synthesized in this study were established on the basis of UV, IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS spectral data. This method for the preparation of chalcones is attractive because it specifically generates the *trans*-isomer (the coupling constants of the two olefinic protons were around 16 Hz), with a high yield. The ease of preparation and the biological activity of chalcones clearly showed that as a class of anticancer agent, they deserved greater attention.[20] Among these, compounds **3**, **8**, **9**, and **10** were prepared for the first time, but the compounds **1**,[21] **2**,[22] **4**–**7**[10,23–27] had been reported earlier. However, as the complete spectral data of some compounds had not been reported so far these were recorded here (vide Experimental).

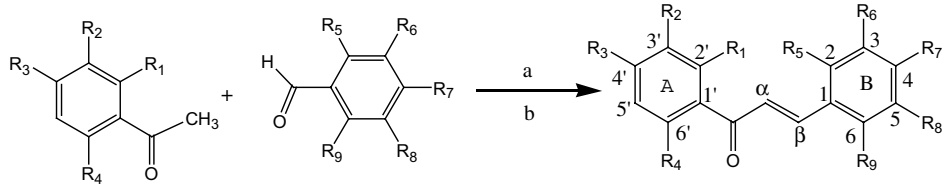
2.2. Effect of 2POCDs on cell proliferation

In a preliminary feasibility study, it was aimed to determine the concentrations of chalcones inhibiting cell growth by 50% (IC_{50}) in two cancer cell lines and in PHA stimulated PBMCs. Cytotoxicity assays were carried out by use of the MTT test. Exponentially growing cultures of each cell line were exposed to a series of increasing concentration of different chalcones. A dose-dependent decrease in cell number was observed in all tested samples demonstrated by IC_{50} values (Table 2). In particular, compounds **1**, **2**, and **8** were the most active agents against Jurkat cell line with IC_{50} values 2.5, 1.7, and 3.2 μM, respectively. Furthermore, compounds **5**–**7** and **10** showed moderate activity ($IC_{50} < 10$ μM), and the remaining compounds were less active toward Jurkat cell line. Similar results were obtained with U937 cell line, among 10 tested compounds, compounds **1**, **2**, and

Table 1. Structure and analytical data of 2′-oxygenated chalcone derivatives



No	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	Formula	Yield (%)
1	OMe	H	OMe	OMe	H	H	H	H	H	$C_{18}H_{18}O_4$	80
2	OH	H	OMe	OMe	OMe	OMe	H	H	H	$C_{18}H_{20}O_6$	88
3	OH	H	OMe	OMe	OMe	OMe	OMe	H	H	$C_{20}H_{22}O_7$	85
4	OH	H	OMe	OMe	OMe	H	OMe	H	OMe	$C_{20}H_{22}O_7$	86
5	OH	H	H	H	OH	H	H	H	H	$C_{15}H_{12}O_3$	45
6	OH	OMe	OMe	H	H	H	OH	H	H	$C_{17}H_{16}O_5$	52
7	OH	H	OH	H	H	OH	OMe	H	H	$C_{16}H_{14}O_5$	46
8	OH	H	OMe	H	OMe	H	H	OMe	H	$C_{18}H_{18}O_5$	77
9	OH	OMe	OMe	H	H	–OCH_2O–		H	H	$C_{18}H_{16}O_6$	82
10	OH	OMe	OMe	H	OMe	OMe	OMe	H	H	$C_{20}H_{22}O_7$	86



Scheme 1. General synthetic route for the synthesis of 2′-oxygenated chalcone derivatives. Reagents and conditions: (a) 60% KOH, EtOH, 48 h; (b) 2 N HCl.

Table 2. Cytotoxicity of chalcone derivatives against human cancer cell lines Jurkat, U937, and PHA stimulated PBMCs [50% inhibition concentration (IC_{50}, μM)]

Compd	Jurkat cells	U937 cells	PBMCs
1	2.5	6.7	6.7
2	1.7	1.5	5.8
3	16.0	16.0	16.0
4	66.8	93.6	107.0
5	8.3	20.8	52.1
6	6.7	13.3	13.3
7	7.0	17.5	35.0
8	3.2	16.0	39.8
9	30.5	30.5	91.5
10	5.3	5.3	10.7

10 were found to exhibit the highest activity with IC_{50} values 6.7, 1.5, and 5.3 μM, respectively; followed by the remaining compounds of less activity. Interestingly, the inhibitory activity values (IC_{50}) of chalcones **2**, **4**, **5**, and **7**–**10** in PBMCs were quite high comparing with the tumor cell line results (Table 2). The data in Table 2 also showed the toxicity profile of 2POCDs and the results implied that these chalcones were more cytotoxic to cancer cell lines than to normal cells.

2.3. Cell cycle analysis

Cell cycle analysis was performed to explore the basis for antiproliferative properties of these synthesized chalcones. For Jurkat cells, at the IC_{50} of each synthesized chalcone, it was found that chalcones **7** and **9** exhibited the highest activity and caused cells arrest in the G2/M phase with a concomitant decrease of cells in the S phase (Table 3). In addition, compounds **5** and **8** showed less activity and cells arrest in the G2/M phase. In contrast, compounds **1** and **2** cause accumulation in the G2/M phase with a concomitant decrease of cells in the S phase. For U937 cells, the results showed at the IC_{50} of compound **3**, it caused marked accumulation in the G0/G1 phase with a concomitant decrease of cells in the S phase (Table 4). However, PHA stimulated normal PBMCs proliferation was inhibited by each synthesized chalcone mostly through arresting cells in the S phase (Table 5).

Table 3. Effect of 2′-oxygenated chalcone derivatives on the distribution of different phases in Jurkat cell cycle

Compd	Concn (μM)	G0/G1	S	G2/M
Control		50.86[a]	23.72	25.42
1	2.5	44.86*	23.08	32.06*
2	1.7	43.75*	24.44	31.81*
3	16.0	46.87	24.57	28.56
4	66.8	49.35	20.61	30.03
5	8.3	49.23	17.78*	32.98*
6	6.7	49.75	20.23	30.02
7	7.0	54.1	0***	45.9**
8	3.2	49.19	16.5*	34.31*
9	30.5	44.03*	0***	55.97**
10	5.3	47.61	20.82	31.57

[a] Percentage of cells in different phases.
* Represents $p < 0.05$ compared with control.
** Represents $p < 0.01$ compared with control.
*** Represents $p < 0.001$ compared with control.

Table 4. Effect of 2′-oxygenated chalcone derivatives on the distribution of different phases in U937 cell cycle

Compd	Concn (μM)	G0/G1	S	G2/M
Control		32.61[a]	43.11	24.28
1	6.7	39.01	0***	60.99***
2	1.5	36.12	19.02**	44.86**
3	16.0	98.27***	1.73***	0***
4	93.6	48.04	33.98	17.98
5	20.8	29.84	16.84**	53.32**
6	13.3	45.45	1.93***	52.63**
7	17.5	42.88	2.49***	54.65**
8	16.0	43.84	5.58***	50.59**
9	30.5	44.14	0***	55.86**
10	5.3	36.82	0***	63.18***

[a] Percentage of cells in different phases.
* Represents $p < 0.05$ compared with control.
** Represents $p < 0.01$ compared with control.
*** Represents $p < 0.001$ compared with control.

Table 5. Effect of 2′-oxygenated chalcone derivatives on the distribution of different phases in PHA stimulated PBMCs cell cycle

Compd	Concn (μM)	G0/G1	S	G2/M
Control		57.67[a]	30.93	11.4
PHA only		92.03	3.63	4.35
1	6.7	54.62**	45.38***	0*
2	5.8	51.35**	36.04**	12.61*
3	16.0	58.73**	27.41**	13.86*
4	107.0	62.37**	37.34**	0.29*
5	52.1	78.33*	17.94*	3.76
6	13.3	72.13*	23.16**	4.53
7	35.0	63.02**	25.76**	11.22*
8	39.8	58.21**	41.79***	0*
9	91.5	71.18*	28.54**	0.29*
10	10.7	34.61***	58.56***	6.84*

[a] Percentage of cells in different phases.
* Represents $p < 0.05$ compared with PHA only.
** Represents $p < 0.01$ compared with PHA only.
*** Represents $p < 0.001$ compared with PHA only.

3. Discussion

Many diverse phytochemicals exist in nature, and a great number of them have been traditionally used for treating specific diseases.[28–31] Massive screening as well as chemical modification of phytochemicals would lead to the discovery of some modern drugs. However, it was often hampered by the difficulty of obtaining a sufficient amount of the phytochemicals. Here, the 2POCDs are also a group of phytochemicals, which occur in very small quantities in nature. Moreover, these compounds have anticancer potential to interfere with targets makes them interesting tools in cancer research.[4–7,14,18,34] They were therefore chemically synthesized, purified, and characterized for investigating their biological activity. The synthesis was easy, one-step reaction, no need for protection of hydroxyl groups and had a good yield. The synthesized compounds were structurally identical to 2POCDs in plants. From the IC_{50} values summarized in Table 2, compounds **1**, **2**, and **8** showed significant ($IC_{50} < 4$ μM) cytotoxic activity, followed by compounds **5**–**7** and **10**, especially selectivity against Jurkat cell lines. Compound **2**, recently reported as natural compound

from the aerial parts of *Caesalpinia pulcherrima*[22] contained a 2′-hydroxy, and methoxyl moieties at the 2-, 3-, 4′-, 6′-positions was the most active in this study toward cancer cell line. Our results provide further support to the recent findings, that 2-hydroxychalcone and 2′-hydroxychalcone were shown to inhibit proliferation of HeLa cells and were the most potent among many other chalcones and related flavonoid derivatives tested.[32] Furthermore, noticed that in all cases 2POCDs were more potent against cancer cell line than corresponding flavanones,[14] and the methoxy group at the carbon-2 atom greatly enhanced the ability of chalcone to inhibit the cell growth. In addition, it is noteworthy that the related chalcone isoliquiritigenin (2′,4,4′-trihydroxychalcone), which occurs naturally in bark and wood of some leguminous trees, was found to be a very strong antitumor agent.[33] The data in this study indicate that the cancer cell lines are more sensitive to the compounds **1**–**3**, **5**–**8**, and **10**, while PHA stimulated PBMCs are relatively resistant to these compounds. In the presence of these compounds, cell growth of the human cancer cell lines Jurkat, and U937 is inhibited, which is indicated by the range of low IC_{50} values from 1.7 to 17.5. Cell growth in the normal PHA stimulated PBMCs is less inhibited, shown by higher IC_{50} values ranging from 5.8 to 52.1. Among the 2POCDs, compounds **4** and **9** with 2,4,6-trimethoxy and 3,4-methylenedioxy moieties in B ring were practically showed less cytotoxicity. It may be due to the significant steric effects induced by the presence of methoxyl group in 2-, 6-positions of compound **4** on ring B. The presence of *ortho*-substituents on ring A, which affect the planarity of compound **9**, may be the cause for its lower activity, but its IC_{50} (30.5 μM), value toward tumor cell lines is lower than the structurally similar compound, 2′-hydroxy-3,3′,4,4′-tetramethoxychalcone IC_{50} (40.2 μM), value against phospholipase A_2.[34]

Several studies demonstrated an association between cell cycle regulation and cancer.[35] The cell cycle machinery regulates cell proliferation, and dysregulated cellular proliferation is a hallmark of cancer.[36,37] Many synthetic cell cycle inhibitors such as flavopiridol, olomoucine, roscovitine, and puvalanol B were viewed as a new generation of anticancer drugs, and some were investigated in clinical trials.[38–40] Cell cycle analysis of 2POCDs treated Jurkat cells clearly showed a G2/M arrest by the compounds **7** and **9**, no effect by the compound **3**, and only a slight effect by the compounds **5** and **8** (Table 3). The increase in the G2/M was accompanied with a decrease of cell numbers in the S phase. The compounds **7**, **9** and **5**, **8** caused G2/M arrest was irreversible. Whereas in the case of U937, compound **3** showed a G0/G1 arrest accompanied by a decrease of cells in the S phase. In contrast, compounds **1**, **2**, and **5**–**10** caused cells arrest in the G2/M phase with a concomitant decrease in percentage of cells in the S phase of the cell cycle (Table 4). PHA stimulated PBMCs proliferation also showed similar profiles (Table 5), where compounds **1** and **8** showed the percentage of cells increase in S phase accompanied with decrease in percentage of cells in the G2/M phase. In contrast, compound **5** showed a G0/G1 arrest associated with the decrease of cells in the S phase. Cell cycle arrest may result in cell death (apoptosis or necrosis). In this study, we found that 2POCDs inhibited proliferation of both normal and cancer cells of various histogenetic origin with IC_{50} values. This effect was mainly due to an inhibition of cell proliferation rather than a direct cytotoxic effect, as suggested by the limited amount of dead cells detected in the culture by the trypan blue exclusion test, and was associated with an inhibition of cell cycle progression. However, this conclusion will require in-depth investigation to conclusively determine whether apoptosis or necrosis is the pathway of cell death, and for identification of a nontoxic, potent compound activity at very low concentrations to provide leads toward new therapeutic agents in cancer research.

4. Conclusion

In an attempt to generate novel anticancer structures with significant activities against human cancer cell line but more potent than those in use nowadays, a series of 10 chalcone derivatives were synthesized and evaluated for their cytotoxicity against Jurkat, U937, human tumor cell lines. Compounds **1**, **2**, and **8**; **1**, **2**, and **10** were identified as potent cytotoxic against Jurkat and U937 cell lines, respectively. Further, cell cycle perturbations induced by the chalcones synthesized were also studied on the Jurkat, U937, and PHA stimulated PBMCs cell lines. The present study revealed that these chalcones were potent antiproliferative agents against tumor cell lines without being more cytotoxic to normal cells. The results of this study may find a lead toward the development of new therapeutic agents to fight cancer.

5. Experimental

5.1. Cell

Jurkat (human lymphocytic cell line), and U937 (human monocytic cell line) were obtained from American Type Culture Collection (Rockville, MD), and cultured with complete RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal calf serum, antibiotics, L-glutamine. The cell numbers were determined with a hemocytometer, and viabilities were assessed by trypan blue dye exclusion. Blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation as described before.[41] The cells were washed twice with Hank's balanced salt solution (HBSS) and resuspended in RPMI 1640 complete medium with 2% human AB serum prior to immunological studies.

5.2. MTT assay

MTT [3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide] assay as described by Mosman[42] was widely used to determine the cytotoxicity of the anticancer drugs. The growth inhibitory activity of the

compounds were determined in the PBMCs and human cancer cell lines by adding MTT into each well and then incubated at 37 °C for 4 h. The MTT formazan crystals (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) formed due to the reduction of MTT, were dissolved by addition of acid–isopropanol and mixed at room temperature. After 20 min, the optical density (OD) was measured with a microplate reader (BIO-RAD, model 3550, USA) at 570 nm ($OD_{570-620}$). The mean OD value of the content of four wells was used for assessing the cell viability. Percent survival was calculated by the following formula

$$(\text{Optical density of drug treated wells}/\text{optical density of untreated wells}) \times 100$$

5.3. Proliferation assay

Proliferation was measured by [^{3}H] thymidine incorporation assay described before.[43] Briefly, different chalcones were added to Jurkat or U937 cell culture of 2×10^6 cells/mL into 96 well microplates for 24 h. PBMCs were stimulated with or without 5 μg/mL phytohemagglutinin-L (PHA) (Sigma) in the presence of chalcones for 72 h. During the last 18 h, cells were pulsed with 1 μCi of [^{3}H] thymidine (Amersham France SA, Les Ulis, France). The result of proliferative response was expressed as (cpm value of experiment)/(cpm value of control) × 100%. The inhibition of 50% proliferation was defined as IC_{50}.

5.4. Cell cycle analysis

Cell cycle analysis was performed by the method described previously.[44,45] Cells were washed in PBS and stained with 20 μg/mL propidium iodide in 0.1% Triton X-100, and 0.1 mM EDTA. Cell suspensions were analyzed with a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). The percentage of cell cycle distribution in the G0/G1, S, and G2/M phases were determined using the MODFIT software (Becton Dickinson).

5.5. Statistical analysis

All experimental data were shown as mean ± SD and, statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnetts post-hoc test, and the significant difference was set at $*p<0.05$; $**p<0.01$.

5.6. Chemistry

Chemicals and solvents were reagent grade and used without further purification. The reactions were monitored by thin layer chromatography (TLC) on precoated Merck Silica gel $60F_{254}$ aluminum plates. Melting points were determined on a Kofler hot stage apparatus. Chemical structures of the compounds as shown in Table 1, were determined by the following spectral data. UV spectra were recorded on a Varian Cary Win UV-50 spectrophotometer. IR spectra were determined in KBr discs on a Perkin–Elmer FT-IR paragon 500 spectrometer. ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.13, 125.43 MHz, respectively, in DMSO-d_6 and $CDCl_3$. ESI-MS were recorded on a Thermo-Finnigan LCQ Advantage system. Column chromatography (CC) separations were carried out by using Silica gel 60 (0.063–0.200 mm) supplied by E. Merck.

General procedure for the synthesis of 2POCDs. 2-Hydroxy-3,4-dimethoxyacetophenone was prepared as reported earlier.[25] All the remaining starting materials were commercially available. To a solution of an appropriately substituted acetophenones (10 mmol), an adecuated benzaldehydes (10 mmol) in EtOH (20 mL) was added a 60% KOH (10 g) solution dropwise at 0 °C in 60 min. The reaction mixture was kept at room temperature for 2 days and then water (50 mL) was added. The mixture was neutralized with 2 N HCl (50 mL) and extracted with EtOAc (3 × 200 mL). The combined organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue yielded was crude chalcone and it was purified by CC eluted with mixtures of hexane/EtOAc to give pure chalcone derivative.

5.6.1. 2′,4′,6′-Trimethoxychalcone (1). This was synthesized using 2,4,6-trimethoxyacetophenone (2.10 g, 10 mmol) and benzaldehyde (1.06 g, 10 mmol) as starting materials: yellow crystalline solid (2.53 g, 80%), mp 110–112 °C; UV λ_{max} (MeOH) (log ε): 305 (3.93), 238 (3.26) nm; IR (KBr) ν_{max}: 2947, 1648, 1601, 1546, 1387 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.65 (2H, m, H-2, H-6), 7.41 (3H, m, H-3, H-4, H-5), 7.21 (1H, d, $J = 16$ Hz, H-β), 6.96 (1H, d, $J = 16$ Hz, H-α), 6.31 (2H, s, H-3′, H-5′); ^{13}C NMR (DMSO-d_6): δ 193.2 (>C=O), 162.0 (C-4′), 158.0 (C-2′, C-6′), 143.5 (C-β), 134.4 (C-1), 130.4 (C-α), 129.2 (C-4), 129.0 (C-2, C-6), 128.4 (C-3, C-5), 91.0 (C-3′, C-5′), 55.8 (2×–OMe), 55.4 (OMe); ESI-MS (positive mode) m/z 299.0 $[M+H]^+$.

5.6.2. 2′-Hydroxy-2,3,4′,6′-tetramethoxychalcone (2). This was synthesized using 2-hydroxy-4,6-dimethoxyacetophenone (1.96 g, 10 mmol) and 2,3-dimethoxybenzaldehyde (1.66 g, 10 mmol) as starting materials: yellow needles (3.19 g, 88%), mp 120–122 °C; UV λ_{max} (MeOH) (log ε): 343 (4.69), 215 (4.23) nm; IR (KBr) ν_{max}: 2937, 1623, 1571, 1449 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 13.40 (1H, br s, OH-2′), 7.86 (1H, d, $J = 16$ Hz, H-β), 7.78 (1H, d, $J = 16$ Hz, H-α), 7.33 (1H, t, $J = 9$ Hz, H-5), 7.15 (2H, d, $J = 9$ Hz, H-4, H-6), 6.16 (1H, d, $J = 2$ Hz, H-5′), 6.13 (1H, d, $J = 2$ Hz, H-3′), 3.89 (3H, s, OMe), 3.88 (3H, s, OMe), 3.82 (3H, s, OMe), 3.78 (3H, s, OMe); ^{13}C NMR (DMSO-d_6): δ 192.4 (>C=O), 165.6 (C-2′), 165.5 (C-4′), 162.0 (C-6′), 152.9 (C-2), 148.1 (C-3), 136.8 (C-β), 128.5 (C-4), 128.3 (C-6), 124.5 (C-α), 119.3 (C-1′), 114.9 (C-1), 96.6 (C-5), 94.0 (C-3′), 91.1

(C-5′), 60.8, 56.2, 55.9, 55.7 (4×–OMe); ESI-MS (positive mode) m/z 345.0 $[M+H]^+$.

5.6.3. 2′-Hydroxy-2,3,4,4′,6′-pentamethoxychalcone (3). This was synthesized using 2-hydroxy-4,6-dimethoxyacetophenone (1.96 g, 10 mmol) and 2,3,4-trimethoxybenzaldehyde (1.96 g, 10 mmol) as starting materials: pale yellow solid (3.33 g, 85%), mp 148–150 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 365 (4.23), 258 (3.16) nm; IR (KBr) ν_{max}: 3260, 1632, 1580, 1555, 1406 cm^{-1}; ^1H NMR (DMSO-d_6): δ 13.57 (1H, br s, OH-2′), 7.80 (1H, d, $J = 16$ Hz, H-β), 7.74 (1H, d, $J = 16$ Hz, H-α), 7.50 (1H, d, $J = 9$ Hz, H-6), 6.91 (1H, d, $J = 9$ Hz, H-5), 6.15 (1H, d, $J = 2.5$ Hz, H-5′), 6.12 (1H, d, $J = 2.5$ Hz, H-3′), 3.89 (3H, s, OMe), 3.86 (3H, s, OMe), 3.84 (3H, s, OMe), 3.82 (3H, s, OMe), 3.77 (3H, s, OMe); ^{13}C NMR (DMSO-d_6): δ 192.4 (>C=O), 165.6 (C-2′), 165.4 (C-4′), 161.8 (C-6′), 155.6 (C-4), 153.0 (C-2), 137.6 (C-3), 126.0 (C-β), 123.8 (C-6), 121.2 (C-α), 108.6 (C-1′), 106.3 (C-1), 99.0 (C-5), 94.0 (C-3′), 91.0 (C-5′), 61.4 (2-OMe), 60.5 (3-OMe), 56.2 (4-OMe), 56.0 (6′-OMe), 55.7 (4′-OMe); ESI-MS (positive mode) m/z 375.0 $[M+H]^+$.

5.6.4. 2′-Hydroxy-2,4,4′,6,6′-pentamethoxychalcone (4). This was synthesized using 2-hydroxy-4,6-dimethoxyacetophenone (1.96 g, 10 mmol) and 2,4,6-trimethoxybenzaldehyde (1.96 g, 10 mmol) as starting materials: orange-red solid (3.37 g, 86%), mp 151–153 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 385 (4.26), 334 (3.28), 256 (3.22) nm; IR (KBr) ν_{max}: 3360, 2900, 1648, 1610, 1503 cm^{-1}; ^1H NMR (DMSO-d_6): δ 14.25 (1H, br s, OH-2′), 8.16 (1H, d, $J = 15.5$ Hz, H-β), 8.11 (1H, d, $J = 16$ Hz, H-α), 6.31 (2H, s, H-3, H-5), 6.13 (1H, d, $J = 2$ Hz, H-5′), 6.09 (1H, d, $J = 2$ Hz, H-3′), 3.91 (6H, s, 2×–OMe), 3.89 (3H, s, OMe), 3.85 (3H, s, OMe), 3.83 (3H, s, OMe); ^{13}C NMR (DMSO-d_6): δ 193.2 (>C=O), 166.5 (C-2′), 163.4 (C-4′), 163.3 (C-6′), 162.0 (C-4), 161.1 (C-2, C-6), 134.4 (C-β), 125.7 (C-α), 106.1 (C-1′), 105.4 (C-1), 94.0 (C-3′), 91.1 (C-3, C-5), 91.0 (C-5′), 56.0 (2×–OMe), 55.9 (OMe), 55.6 (2×–OMe); ESI-MS (positive mode) m/z 375.0 $[M+H]^+$.

5.6.5. 2,2′-Dihydroxychalcone (5). This was synthesized using 2-hydroxyacetophenone (1.36 g, 10 mmol) and salicylaldehyde (1.22 g, 10 mmol) as starting materials: yellow amorphous powder (1.16 g, 45%), mp 156–158 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 368 (3.98), 310 (2.86), 255 (2.39) nm; IR (KBr) ν_{max}: 3406, 3240, 1640 cm^{-1}; ^1H NMR (DMSO-d_6): δ 12.62 (1H, br s, OH-2′), 8.17 (1H, d, $J = 8$ Hz, H-6), 8.16 (1H, d, $J = 15.5$ Hz, H-β), 7.97 (1H, d, $J = 16$ Hz, H-α), 7.90 (1H, d, $J = 8$ Hz, H-6′), 7.55 (1H, dt, $J = 1$, 8.5 Hz, H-4), 7.30 (1H, dt, $J = 1$, 8 Hz, H-5), 6.95–7.10 (2H, m, H-3′, H-5′), 6.89 (1H, dt, $J = 1$, 8.5 Hz, H-4′); ^{13}C NMR (DMSO-d_6): δ 193.8 (>C=O), 161.9 (C-2′), 157.5 (C-2), 140.3 (C-β), 136.1 (C-4), 132.5 (C-6), 130.5 (C-α), 128.9 (C-1), 121.1 (C-5), 120.7 (C-3), 120.2 (C-1′), 119.4 (C-4′), 119.1 (C-5′), 117.7 (C-3′), 115.4 (C-6′); ESI-MS (positive mode) m/z 241.1 $[M+H]^+$.

5.6.6. 2′,4-Dihydroxy-3′,4′-dimethoxychalcone (helianone A) (6). This was synthesized using 2-hydroxy-3,4,-dimethoxyacetophenone (1.96 g, 10 mmol) and *p*-hydroxybenzaldehyde (1.22 g, 10 mmol) as starting materials: yellow crystalline solid (1.65 g, 52%), mp 144–146 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 366 (4.10), 308 (3.30) nm; IR (KBr) ν_{max}: 3382, 1630, 1590 cm^{-1}, ^1H NMR ($CDCl_3$): δ 13.26 (1H, s, OH-2′), 7.78 (1H, d, $J = 16$ Hz, H-β), 7.59 (1H, d, $J = 9$ Hz, H-6′), 7.48 (2H, d, $J = 8.6$ Hz, H-2, H-6), 7.36 (1H, d, $J = 16$ Hz, H-α), 6.78 (2H, d, $J = 8.6$ Hz, H-3, H-5), 6.50 (1H, d, $J = 9$ Hz, H-5′), 5.54 (1H, s, OH-4), 3.91 (3H, s, OMe), 3.89 (3H, s, OMe); ^{13}C NMR ($CDCl_3$): δ 192.4 (>C=O), 158.3 (C-4′), 158.1 (C-2′), 157.9 (C-4), 144.6 (C-β), 136.2 (C-3′), 130.5 (C-2, C-6), 127.0 (C-1), 125.8 (C-1′), 117.4 (C-α), 116.0 (C-3, C-5), 115.2 (C-5′), 102.5 (C-6′), 60.2 (OMe), 56.0 (OMe); ESI-MS (positive mode) m/z 301.1 $[M+H]^+$.

5.6.7. 2′,3,4′-Trihydroxy-4-methoxychalcone (7). This was synthesized using resacetophenone (1.52 g, 10 mmol) and isovanillin (1.52 g, 10 mmol) as starting materials: yellow solid (1.40 g, 46%), mp 196–198 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 370 (4.68), 312 (3.69), 270 (3.74) nm; IR (KBr) ν_{max}: 3420, 3236, 2972, 1665, 1594 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.45 (1H, s, OH-2′), 7.85 (1H, d, $J = 9$ Hz, H-6′), 7.68 (1H, d, $J = 16$ Hz, H-β), 7.50 (1H, d, $J = 16$ Hz, H-α), 7.15 (1H, d, $J = 6$ Hz, H-2), 6.89 (1H, dd, $J = 2$, 8 Hz, H-6), 6.48 (1H, d, $J = 8$ Hz, H-5), 6.31 (1H, dd, $J = 2$, 8 Hz, H-5′), 6.16 (1H, d, $J = 2$ Hz, H-3′), 4.01 (3H, s, OMe); ^{13}C NMR ($CDCl_3$): δ 192.9 (>C=O), 166.4 (C-2′), 162.6 (C-4′), 152.0 (C-4), 150.6 (C-3), 143.8 (C-β), 132.7 (C-6′), 129.2 (C-1), 125.3 (C-6), 116.9 (C-α), 116.0 (C-5), 113.9 (C-1′), 109.5 (C-2), 108.1 (C-5′), 101.8 (C-3′), 58.1 (OMe); ESI-MS (positive mode) m/z 287.1 $[M+H]^+$.

5.6.8. 2′-Hydroxy-2,4′,5-trimethoxychalcone (8). This was synthesized using 2-hydroxy-4-methoxyacetophenone (1.66 g, 10 mmol) and 2,5-dimethoxybenzaldehyde (1.66 g, 10 mmol) as starting materials: orange red crystals (2.56 g, 77%), mp 108–111 °C; UV λ_{max} (MeOH) ($\log \varepsilon$): 372 (3.68), 309 (3.74), 264 (2.97) nm; IR (KBr) ν_{max}: 3218, 2988, 1624, 1592, 1503 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.50 (1H, s, OH-2′), 8.12 (1H, d, $J = 15.5$ Hz, H-β), 7.80 (1H, d, $J = 9$ Hz, H-6′), 7.64 (1H, d, $J = 15.5$ Hz, H-α), 7.14 (1H, s, H-6), 6.93 (1H, d, $J = 7.5$ Hz, H-4), 6.86 (1H, d, $J = 9$ Hz, H-5′), 6.46 (2H, m, H-3, H-3′), 3.87 (3H, s, OMe), 3.84 (3H, s, OMe), 3.80 (3H, s, OMe); ^{13}C NMR ($CDCl_3$): δ 192.6 (>C=O), 166.9 (C-4′), 166.3 (C-2′), 153.8 (C-2), 140.1 (C-5), 131.5 (C-β), 124.6 (C-6), 121.5 (C-α), 117.5 (C-1′), 114.5 (C-4), 114.2 (C-5′), 112.7 (C-1), 107.9 (C-3), 101 (C-6′), 97.2 (C-3′), 56.4 (OMe), 56.0 (OMe), 55.7 (OMe); ESI-MS (positive mode) m/z 315.1 $[M+H]^+$.

5.6.9. 2′-Hydroxy-3′,4′-dimethoxy-3,4-methylenedioxychalcone (9). This was synthesized using 2-hydroxy-3,4-dimethoxyacetophenone (1.96 g, 10 mmol) and piperonal (1.50 g, 10 mmol) as starting materials: pale yellow

crystals (2.84 g, 82%), mp 174–176 °C; UV λ_{max} (MeOH) (log ε): 371 (4.57), 318 (sh) (3.14), 258 (3.10) nm; IR (KBr) ν_{max}: 3362, 2875, 1627, 1554, 1445 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.25 (1H, s, OH-2′), 7.79 (1H, d, $J = 15$ Hz, H-β), 7.64 (1H, d, $J = 8.5$ Hz, H-6′), 7.38 (1H, d, $J = 15$ Hz, H-α), 7.15 (1H, s, H-2), 7.11 (1H, d, $J = 8.5$ Hz, H-6), 6.83 (1H, d, $J = 8.5$ Hz, H-5), 6.50 (1H, d, $J = 8$ Hz, H-5′), 6.01 (2H, s, –OCH_2O–), 3.93 (3H, s, OMe), 3.88 (3H, s, OMe); ^{13}C NMR ($CDCl_3$): δ 192.5 (>C=O), 158.9 (C-2′), 158.5 (C-4′), 150.4 (C-4), 148.7 (C-3), 144.8 (C-3′), 137.0 (C-β), 129.4 (C-1), 126.1 (C-6), 125.8 (C-α), 118.4 (C-5), 115.9 (C-1′), 109.0 (C-1), 106.9 (C-2), 103.0 (C-6′), 101.9 (–OCH_2O–), 60.9 (OMe), 56.4 (OMe); ESI-MS (positive mode) m/z 329.1 $[M+H]^+$.

5.6.10. 2′-Hydroxy-2,3,3′,4,4′-pentamethoxychalcone (10). This was synthesized using 2-hydroxy-3,4-dimethoxyacetophenone (1.96 g, 10 mmol) and 2,3,4-trimethoxybenzaldehyde (1.96 g, 10 mmol) as starting materials: yellow solid (3.38 g, 86%), mp 98–100 °C; UV λ_{max} (MeOH) (log ε): 365 (4.12), 308 (sh) (3.35), 236 (3.32) nm; IR (KBr) ν_{max}: 3214, 2896, 1634, 1595, 1487 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.34 (1H, s, OH-2′), 8.03 (1H, d, $J = 16$ Hz, H-β), 7.66 (1H, d, $J = 9$ Hz, H-6′), 7.60 (1H, d, $J = 15.5$ Hz, H-α), 7.35 (1H, d, $J = 8.5$ Hz, H-6), 6.70 (1H, d, $J = 8.5$ Hz, H-5), 6.50 (1H, d, $J = 9$ Hz, H-5′), 3.94 (3H, s, OMe), 3.93 (3H, s, OMe), 3.92 (3H, s, OMe), 3.89 (3H, s, OMe), 3.87 (3H, s, OMe); ^{13}C NMR ($CDCl_3$): δ 193.1 (>C=O), 158.6 (C-4′), 158.5 (C-2′), 156.3 (C-4), 154.2 (C-2), 142.7 (C-3), 140.4 (C-3′), 137.0 (C-β), 126.1 (C-α), 124.6 (C-6), 122.0 (C-1′), 119.6 (C-1), 116.0 (C-5′), 107.8 (C-5), 103.0 (C-6′), 61.6, 61.1, 60.9 (3×–OMe), 56.3 (2×–OMe); ESI-MS (positive mode) m/z 375.1 $[M+H]^+$.

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References and notes

1. Park, E. J.; Park, H. R.; Lee, J. S.; Kim, J. *Planta Med.* **1998**, *64*, 464.
2. De Vincenzo, R.; Scambia, G.; Benedetti Panici, P.; Ranelletti, F. O.; Bonanno, G.; Ercoli, A.; Delle Monache, F.; Ferrari, F.; Piantelli, M.; Mancuso, S. *Anticancer Drug Des.* **1995**, *10*, 481.
3. Rui, H. *J. Cell. Biochem.* **1997**, *67*, 7.
4. Claude, A. C.; Jean, C. L.; Patric, T.; Christelle, P.; Gerard, H.; Albert, J. C.; Jean, L. D. *Anticancer Res.* **2001**, *21*, 3949.
5. Rosa, D. V.; Cristiano, F.; Mariagrazia, D.; Cristiana, G.; Antonella, R.; Ezio, B.; Paolo, M.; Piero, V.; Federica, B.; Franco, O. R.; Salvatore, M.; Giovanni, S. *Cancer Cheomother. Pharmacol.* **2000**, *46*, 305.
6. Kumar, S. K.; Erin, H.; Catherine, P.; Halluru, G.; Davidson, N. E.; Khan, S. R. *J. Med. Chem.* **2003**, *46*, 2813.
7. Anto, R. J.; Sukumaran, K.; Kuttan, G.; Rao, M. N. A.; Subbaraju, V.; Kuttan, R. *Cancer Lett.* **1995**, *97*, 33.
8. Ko, H. H.; Tsao, L. T.; Yu, K. L.; Liu, C. T.; Wang, J. P.; Lin, C. N. *Bioorg. Med. Chem.* **2003**, *11*, 105.
9. Matsuda, H.; Morikawa, T.; Ando, S.; Iwao, T.; Masayuki, Y. *Bioorg. Med. Chem.* **2003**, *11*, 1995.
10. Hsieh, H. K.; Tsao, L. T.; Wang, J. P.; Lin, C. N. *J. Pharm. Pharmacol.* **2000**, *52*, 163.
11. Parmer, V. S.; Sharma, N. K.; Husain, M.; Watterson, A. C.; Kumar, J.; Samuelson, L. A.; Ashok, L. C.; Prasad, A. K.; Kumar, A.; Malhotra, S.; Kumar, N.; Jha, A.; Singh, A.; Singh, I.; Himanshu; Vats, A.; Shakil, N. A.; Trikha, S.; Mukherjee, S.; Sharma, S. K.; Singh, S. K.; Kumar, A.; Jha, H. N.; Olsen, C. E.; Stove, C. P.; Bracke, M. E.; Mareel, M. M. *Bioorg. Med. Chem.* **2003**, *11*, 913.
12. Lin, Y. M.; Zhou, Y.; Flavin, M. T.; Zhou, L. M.; Nie, W.; Chen, F. C. *Bioorg. Med. Chem.* **2002**, *10*, 2795.
13. Lopez, S. N.; Castelli, M. V.; Zacchino, S. A.; Dominguez, J. N.; Lobo, G.; Jaime, C. C.; Cortes, J. C. G.; Ribas, J. C.; Devia, C.; Ana, M. R.; Ricardo, D. E. *Bioorg. Med. Chem.* **2001**, *9*, 1999.
14. Pouget, C.; Lauthier, F.; Simon, A.; Fagnere, C.; Basly, J. P.; Delage, C.; Chulia, A. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3095.
15. Kim, Y. P.; Ban, H. S.; Lim, S. S.; Kimura, N.; Jung, S. H.; Ji, J.; Lee, S.; Ryu, N.; Keum, S. R.; Shi, K. H.; Ohuchi, K. *J. Pharm. Pharmacol.* **2001**, *53*, 1295.
16. Huang, Y. C.; Guh, J. W.; Cheng, Z. J.; Chang, Y. L.; Hwang, T. L.; Lin, C. N.; Teng, C. M. *Life Sci.* **2001**, *68*, 2435.
17. Stevens, J. F.; Miranda, C. L.; Frei, B.; Buhler, D. R. *Chem. Res. Toxicol.* **2003**, *16*, 1277.
18. Xia, Y.; Yang, Z. Y.; Xia, P.; Bastow, K. F.; Nakanishi, Y.; Lee, K. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 699.
19. Wattanasin, S.; Murphy, W. S. *Synthesis* **1980**, 647.
20. Devia, A. C.; Ferretti, F. H.; Ponce, C. A.; Tomas, F. *J. Mol. Struct. (TEOCHEM)* **1999**, *493*, 187.
21. Schmid, R. D.; Mues, R.; Mc Reynolds, J. H.; Velde, G. V.; Nakatani, N.; Rodriguez, E.; Mabry, T. J. *Phytochemistry* **1973**, *12*, 2765.
22. Srinivas, K. V. N. S.; Rao, Y. K.; Mahender, I.; Das, B.; Krishna, K. V. S. R.; Kishore, K. H.; Murthy, U. S. N. *Phytochemistry* **2003**, *63*, 789.
23. Tokunaru, H.; Yasuhiko, K.; Chikako, S.; Ayako, A.; Masafumi, K. *J. Chem. Soc., Perkin Trans. 1* **1994**, *6*, 753.
24. Albena, T. D. K.; Chitrananda, A.; Paul, T. *J. Med. Chem.* **1998**, *41*, 5287.
25. Rao, Y. K.; Rao, C. V.; Kishore, P. H.; Sekar, D. G. *J. Nat. Prod.* **2001**, *64*, 368.
26. Macias, F. A.; Molinillo, J. M. G.; Torres, A.; Varela, R. M.; Castellano, D. *Phytochemistry* **1997**, *45*, 683.
27. Maldonado, E.; Bello, M.; Villasenor, J. L.; Ortega, A. *Phytochemistry* **1998**, *49*, 1115.
28. Mans, D. R. A.; Rocha, A. B. D.; Schwartsmann, G. *Oncologist* **2000**, *5*, 185.
29. Barker, A. J.; Gibson, K. H.; Grudy, W.; Godfrey, A. A.; Barlow, J. J.; Healy, M. P.; Woodburn, J. R.; Ashton, S. E.; Curry, B. J.; Scarlett, L.; Henthorn, L.; Richards, L. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1911.
30. Mehrotra, S.; Singh, V. K.; Agarwal, S. S.; Maurya, R.; Srimal, R. C. *Exp. Mol. Pathol.* **2002**, *72*, 236.
31. Karkabounas, S.; Assimakopoulos, D.; Malamas, M.; Skaltsounis, A. L.; Leonce, S.; Zelovitis, J.; Stefanou, D.; Evangelou, A. *Anticancer Res.* **2000**, *20*, 4391.
32. Ramanathan, R.; Das, N. P.; Tan, C. H. *Int. J. Oncol.* **1993**, *3*, 115.

33. Yamamoto, S.; Aizu, E.; Jiang, H.; Nakadate, T.; Kiyoto, I.; Wang, J. C.; Kato, R. *Carcinogenesis* **1991**, *12*, 317.
34. Ballesteros, J. F.; Sanz, M. J.; Ubeda, A.; Miranda, M. A.; Iborra, S.; Paya, M.; Alcaraz, M. J. *J. Med. Chem.* **1995**, *38*, 2794.
35. Hajduch, M.; Havlieek, L.; Vesely, J.; Novotny, R.; Mihal, V.; Strand, M. *Adv. Exp. Med. Biol.* **1999**, *457*, 341.
36. Paggi, M. G.; Baldi, A.; Bonetto, F.; Giordano, A. *J. Cell. Biochem.* **1996**, *62*, 418.
37. Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57.
38. Buolamwini, J. K. *Curr. Pharm. Res.* **2000**, *6*, 379.
39. Stadler, W. M.; Volgelzang, N. J.; Amato, R.; Sosman, J.; Taber, D.; Liebowitz, D.; Vokes, E. E. *J. Clin. Oncol.* **2000**, *18*, 371.
40. Senderowicz, A. M.; Headlee, D.; Stinson, S. F.; Lush, R. M.; Kalil, N.; Villalba, L.; Hill, K.; Steinberg, S. M.; Figg, W. D.; Tompkins, A.; Arbuck, S. G.; Sausville, E. A. *J. Clin. Oncol.* **1998**, *16*, 2986.
41. Fang, S. H.; Chiang, B. L.; Wu, M. H.; Iba, H.; Lai, M. Y.; Yang, P. M.; Chen, D. S.; Hwang, L. H. *J. Clin. Microbiol.* **2001**, *39*, 3895.
42. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
43. Fang, S. H.; Lai, M. Y.; Hwang, L. H.; Yang, P. M.; Chen, P. J.; Chiang, B. L.; Chen, D. S. *J. Biomed. Sci.* **2001**, *8*, 484.
44. Gomez-Angelats, M.; Bortner, C. D.; Cidlowski, J. A. *J. Biol. Chem.* **2000**, *275*, 19609.
45. Grana, X.; Reddy, E. P. *Oncogene* **1995**, *11*, 211.