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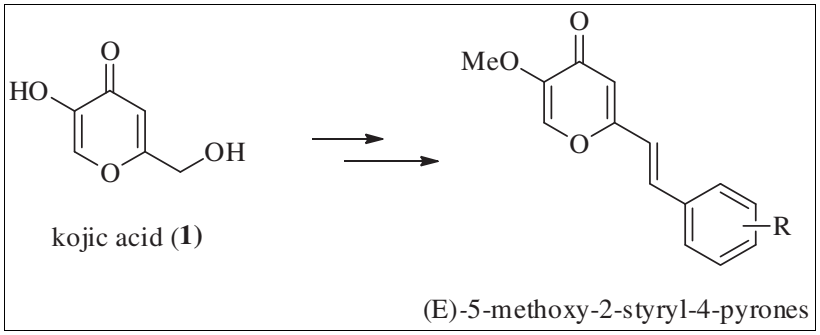

The present study a series of (E)-5-methoxy-2-styryl-4*H*-pyran-4-ones **6a–j** was synthesized and evaluated for growth inhibitory inhibition against carcinoma cells. The growth inhibition study of eight carcinoma cell lines was examined and demonstrated that SKHep cells exhibit significant structure-activity relationship in response to the tested compounds. Among them, **6f** showed the most potent activity against SKHep, A549, AGS, and H460 cell lines with GI_{50} values of 0.17, 8.3, 3.6, 8.0 μM, respectively.

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INTRODUCTION

Kojic acid (**1**) is a metabolic compound produced by several species of fungi, such as *Aspergillus*, *Acetobacter*, and *Penicillium* [1], Figure 1. It has been added to food as an antioxidant [2], as a preservative to prevent formation of warm-over flavor in beef [3]. Additionally, it serves as a food additive for preventing enzymatic discoloration in vegetables, crabs, and shrimps [4,5] and as a skin lightening or bleaching agent in cosmetic preparations [6,7]. Among its pharmacological activities, kojic acid has been demonstrated to exhibit bacteriostatic activity [8], anti-inflammatory effects [9], insecticidal activity [10], antibiotic activity [11], and cytotoxic and antitumor properties [12,13]. Particularly, kojic acid accessibly carried out-various structural modifications, thanks to its flexibility and amenability. For instance, both ionizable and nonionizable derivatives of kojic acid have shown to exhibit growth inhibitory effect against *Escherichia coli* [14]. Selenium containing kojic acid derivatives have showed cytotoxic effects against carcinoma cells [15]. Mimosine, a ring isomer of kojic acid, has revealed superior antiproliferative effects to the parent compound [16]. Moreover, organomercury (II) complexes of kojic acids also demonstrated improved antibacterial activity compared with kojic acid [17]. As such, the scaffold of kojic acid shows commitment to be a promising lead for structural modifications aimed at developing potential drug-like small molecules. To the best of our knowledge, (E)-5-methoxy-2-styryl-4-pyrones derived from kojic acid have not yet been studied for their anticancer activity. Herein, we take advantage of kojic acid as a molecular template to synthesize a series of (E)-5-methoxy-2-styryl-4*H*-pyran-4-ones and to examine their growth inhibitory effect on a panel of carcinoma cell lines as shown in Figure 1.

RESULTS AND DISCUSSION

Synthesis. The synthesis of (E)-5-methoxy-2-styryl-4*H*-pyran-4-ones is outlined in Scheme 1. As shown, the C-5 hydroxyl group in kojic acid **(1)** was selectively methylated by treatment of dimethyl sulfate (Me_2SO_4) in the presence of 10% potassium hydroxide (KOH) to afford 2-(hydroxymethyl)-5-methoxy-4*H*-pyran-4-one **2** in 70% isolated yield [18]. The hydroxyl group on 2-methylene moiety in **2** was further subjected to undergo mesylation with mesyl chloride (MsCl) in the presence of triethylamine (NEt_3) in dichloromethane (DCM) to yield mesylate ester **3**. Without further purification, the nucleophilic substitution of the mesylate ester **3** and sodium bromide (NaBr) was carried out in DMF to generate 2-(bromomethyl)-5-methoxy-4*H*-pyran-4-one **4** in 63% isolated yield in two steps. Furthermore, the Arbuzov

reaction of 2-(bromomethyl)-5-methoxy-4*H*-pyran-4-one **4** and triethyl phosphite ($P(OEt)_3$) in toluene at reflux gave phosphonate **5** in 68% isolated yield. Having the phosphonate **5** in hand, a series of (E)-5-methoxy-2-styryl-4-pyrones **6a–j** was successfully synthesized by the Horner–Emmons reaction, in which the phosphonate **5** and various benzaldehydes underwent olefination reaction mediated by the base sodium hydride (NaH) in the anhydrous THF solution [19,20].

Growth inhibition. The evaluation of growth-inhibitory effect of **6a–j** was examined in a panel of eight human carcinoma cell lines, including SKHep (hepatocellular carcinoma cell), A549 (non-small cell lung adenocarcinoma cell), AGS (gastric adenocarcinoma cell), PC-3 (prostate carcinoma cell), H460 (lung large cell carcinoma cell), SW620 (colorectal adenocarcinoma cell), HeLa (cervical epithelioid carcinoma cell), and OVCA (ovarian carcinoma cell. All cell lines were exposed to the tested compounds at indicated concentration in 10% fetal bovine serum (FBS)-supplemented Dulbecco's Modified Eagle Medium for a 48-h treatment. The indicated growth inhibition in response to the tested compounds was employed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Surprisingly, only SKHep cells exhibited significant growth inhibition in response to **6a–j** treatment with a range of GI_{50} values between 0.17 and 36.1 μM. As shown in Table 1, the tested compounds bearing electron-donating groups on the benzene ring such as 4′-methoxy (**6c**, GI_{50}, 0.61 μM), 3′,4′-dimethoxy (**6d**, GI_{50}, 0.24 μM), 3′,5′-dimethoxy (**6e**, GI_{50}, 0.18 μM), and 3′,4′,5′-trimethoxy (**6f**, GI_{50}, 0.17 μM) displayed remarkable growth-inhibitory effect on SKHep cells compared with **6a** (GI_{50}, 5.0 μM), indicating that electronic effect of tested compounds play a critical for activity. Moreover, we found that **6c** (GI_{50}, 0.61 μM) containing 4-methoxy group on the benzene ring exhibited 4.6-fold in potency compared with its counterpart **6b** (GI_{50}, 2.8 μM) with 3-methoxy group. This finding suggests that an appropriate position of substituent attached to the tested compound is significantly improved to exhibit interactions with its not yet indentified targets for growth inhibition. Compared with its counterpart **6c** (GI_{50}, 0.61 μM), **6j** bearing an electron-donating 4-*N*, *N*-dimethylamino group showed less potent activity with a GI_{50} value of 2.6 μM. On the contrary, introduction of electron-withdrawing groups on the benzene ring such as 4-trifluoromethyl (**6h**, GI_{50}, 36.1 μM) and 4-trifluoromethoxy (**6i**, GI_{50}, 4.7 μM) groups resulted in a precipitate decrease in potency. According to such interesting findings, we concluded that SKHep cells clearly exhibited structure-activity relationship in response to our tested compounds. On the other hand, upon exposure of OVCA cells to the tested compounds, only **6c**, **6d**, **6e**, **6f** and **6j** showed moderate growth inhibitory effect with GI_{50} values of 19.4, 26.4, 17.8, 18.1 and 21.3 μM, respectively. Except for **6f** with moderate activity, all tested compounds did not revealed appreciable growth-inhibitory effect against the rest of six cell lines as high as 40 μM treatment. As indicated in Table 2, compound **6f** bearing 3′,4′,5′-trimethoxy groups exhibited an appreciable growth inhibitory activity with GI_{50} values of 8.3, 3.6, 8.0 μM against A549, AGS and H460 cell lines, respectively. Taken together, we clearly summarized that individual cell lines indeed revealed a distinct sensitivity in response to these compounds.

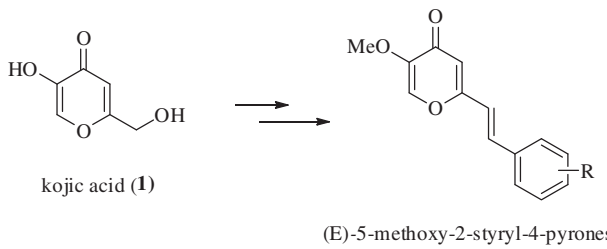
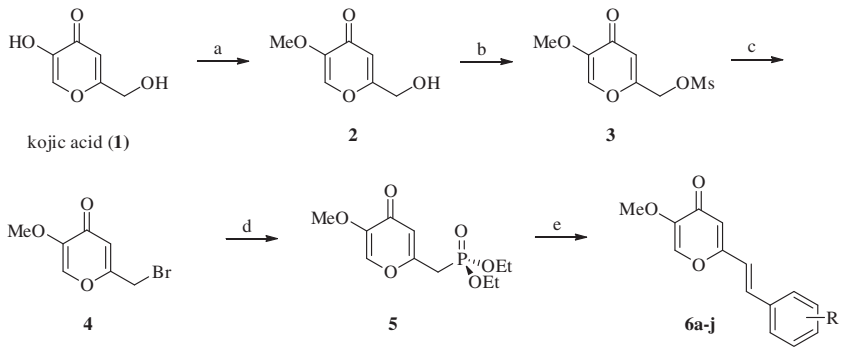


Figure 1. Chemical structures of kojic acid (**1**) and its derivatives.

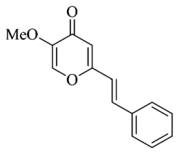
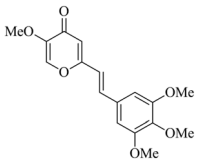
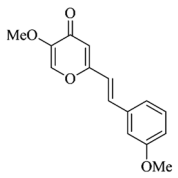
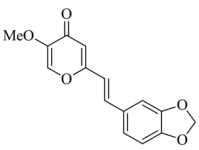
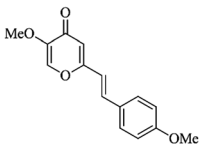
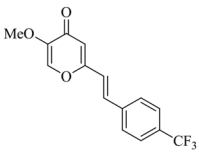
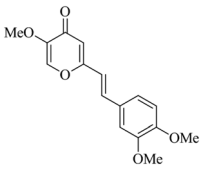
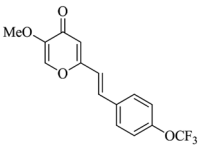
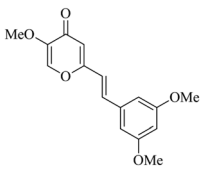
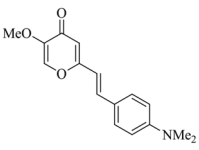
Scheme 1. Preparation of (E)-5-methoxy-2-styryl-4*H*-pyran-4-ones **6a–j**.



Reagents and conditions: a) Me_2SO_4 , 10% KOH; b) MsCl, NEt_3, DCM, 0°C; c) NaBr, DMF, rt; d) $P(OEt)_3$, toluene, reflux; e) ArCHO, NaH, THF, 0°C to rt.

Table 1

Chemical structures and growth inhibitory effect of (E)-5-methoxy-2-styryl-4-pyrones **6a–j** against carcinoma cells.

Entry	Structure	GI_{50}[a] (μM)		Entry	Structure	GI_{50}[a] (μM)	
		SKHep	OVCA			SKHep	OVCA
6a		5.0 ± 0.4	>40	**6f**		0.17 ± 0.01	18.1 ± 2.2
6b		2.8 ± 0.2	>40	**6g**		2.9 ± 0.5	>40
6c		0.61 ± 0.34	19.4 ± 1.8	**6h**		36.1 ± 1.5	>40
6d		0.24 ± 0.05	26.4 ± 2.2	**6i**		4.7 ± 1.1	>40
6e		0.18 ± 0.02	17.8 ± 1.2	**6j**		2.6 ± 0.7	21.3 ± 2.1

OVCA, Ovarian carcinoma cell.
[a]GI_{50} values are presented as the mean ± standard error of the mean from four to six separated experiments.

Table 2

GI_{50} values of (E)-5-methoxy-2-(3′, 4′, 5′-trimethoxy)styryl-4-pyrone **6f** on a panel of human cancer cell lines.

Cell line	Tumor type	GI_{50}[a] (μM)	
		6f	Doxorubicin
SKHep	Hepatocellular carcinoma cell	0.17 ± 0.01	0.09 ± 0.03
A549	Non-small cell lung adenocarcinoma cell	8.3 ± 0.3	2.23 ± 0.3
AGS	Gastric adenocarcinoma cell	3.6 ± 0.3	0.02 ± 0.01
PC-3	Prostate carcinoma cell	21.4 ± 4.3	1.76 ± 0.71
H460	Lung large cell carcinoma cell	8.0 ± 1.4	0.11 ± 0.02
SW620	Colorectal adenocarcinoma cell	11.8 ± 2.8	0.03 ± 0.005
HeLa	Cervical epithelioid carcinoma cell	28.1 ± 2.6	0.33 ± 0.04
OVCA	Ovarian carcinoma cell	18.1 ± 2.2	0.95 ± 0.07

[a]GI_{50} values are presented as the mean ± SEM (standard error of the mean) from four to six separated experiments.

CONCLUSION

The present study we take advantage of kojic acid as a lead to synthesize a series of (E)-5-methoxy-2-styryl-4 *H*-pyran-4-ones **6a-j**. The growth inhibition study of eight carcinoma cell lines was examined and demonstrated that the SKHep cells exhibited a significant structure-activity relationship in response to the tested compounds. Particularly, compound **6f** showed the most potent activity against SKHep, A549, AGS, and H460 cell lines with GI_{50} values of 0.17, 8.3, 3.6, 8.0 μM, respectively. In summary, structural modifications of kojic acid have been carried out and examined to show highly selective growth inhibition on SKHep cells that pave the way for further mechanistic study.

EXPERIMENTAL

Chemistry. Chemical reagents and organic solvents were purchased from TCI and Alfa Aesar unless otherwise mentioned. Nuclear magnetic resonance spectra (^{1}H-NMR and ^{13}C-NMR) were measured on a Bruker AC-300 instrument purchased from Bruker Daltonics Inc. (Fremont, CA). Chemical shifts (δ) are reported in ppm relative to the TMS peak. High resolution mass spectra (HRMS) were obtained by fast atom bombardment (FAB) on a Jeol JMS-700 instrument. Flash column chromatography was performed with Merck Kiesegel 60 Art (230–400 mesh).

2-(Hydroxymethyl)-5-methoxy-4H-pyran-4-one (2). To a well-stirred solution of kojic acid (**1**) (2.5 g, 17.6 mmol) and in 10% potassium hydroxide (20 mL), redistilled dimethyl sulfate (2.5 g, 20.09 mmol) was added. The reaction mixture was kept below 25°C by occasional cooling in an ice-bath. Stirring was continued for an additional 30 min and the mixture was cooled in an ice-bath and filtered, followed by proper recrystallization from methanol to obtain **2**. Yield: 70 %. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.13 (s, 3H), 4.30 (d, J = 5.8 Hz, 2H), 5.70 (t, J = 5.8 Hz, 1H), 6.32 (s, 1H), 8.16 (s, 1H) ppm.

(5-Methoxy-4-oxo-4H-pyran-2-yl)methyl methanesulfonate (3). To a stirred solution of **2** (1.56 g, 10.0 mmol) in DCM (20 mL), triethylamine (1.01 g, 10.0 mmol) and methanesulfonyl chloride (1.13 g, 10.0 mmol) was added at 0°C. After stirring for 30 min at an ice-bath, the mixture was diluted with ethyl acetate (20 mL) and washed with brine (20 mL). The organic layer was dried over $MgSO_4$, evaporated *in vacuo* to obtain crude **3**. Yield: 90%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.15 (s, 3H), 3.32 (s, 3H), 5.16 (s, 2H), 6.57 (s, 1H), 8.27 (s, 1H) ppm.

2-(Bromomethyl)-5-methoxy-4H-pyran-4-one (4). To a stirred solution of **3** (2.23 g, 9.0 mmol) in DMF (15 mL), NaBr (1.02 g, 10.0 mmol) was added, and the mixture was stirred at room temperature for 20 min. As excess amount of NaBr was filtered, the filtrate was diluted with ethyl acetate (30 mL) and washed with water (25 mL) and brine (20 mL). The organic layer was dried over $MgSO^4$, concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate/dichloromethane = 3/7) to obtain **4**. Yield:72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.15 (s, 3H), 5.08 (s, 2H), 6.47 (s, 1H), 7.56 (s, 1H) ppm.

Diethyl (5-methoxy-4-oxo-4H-pyran-2-yl)methylphosphonate (5). To a solution of **4** (1.1 g, 5.0 mmol) in toluene (15 mL), triethyl phosphite (3.32 g, 20.0 mmol) was added, and the mixture was heated at reflux. After 96 h, excess amount of triethyl phosphite was removed *in vacuo* and the resulting oily residue was purified by flash column to afford **5**. Yield: 68%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (t, J = 7.1 Hz, 6H), 3.01 (s, 1H), 3.08 (s, 1H), 3.75(s, 3H), 4.11 (q, J = 7.1 Hz, 4H), 6.31(s, 1H), 7.52 (s, 1H) ppm.

General procedure for the synthesis of 5-methoxy-2-styryl-4-pyrones. (E)-5-Methoxy-2-styryl-4H-pyran-4-one (6a). To a stirred solution of **5a** (0.276 g, 1.0 mmol) and NaH (0.08 g, 2.0 mmol, 60% suspension in mineral oil) in dry THF (20 mL), a solution of benzaldehyde (0.106 g, 1.0 mmol) in THF (5 mL) was added dropwise under Argon atmosphere at 0°C. After stirring at room temperature for 1 h, the mixture was diluted with dichloromethane (20 mL) and washed with water (40 mL) and brine (40 mL). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate/dichloromethane = 1/9) to afford **6a**. Yield: 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.77(s, 3H), 6.36(s, 1H), 6.66(d, J = 16.2 Hz, 1H), 7.32 (d, J = 16.2 Hz, 1H), 7.33–7.39(m, 3H), 7.49 (d, J = 9.7 Hz, 2H), 7.56(s, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl^3$) δ 56.6, 113.3, 119.4, 127.7, 129.1, 129.9, 135.0, 136.4, 137.3, 148.8, 161.5, 174.5 ppm. HRMS $(M+1)^+$ Calcd for $C_{14}H_{12}O_3$ 228. 228.2433; found 228.2431.

(E)-5-methoxy-2-(3-methoxystyryl)-4H-pyran-4-one (6b). The synthesis of **6b** was similar to that of **6a**. Yield: 81%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80(s, 3H), 3.85(s, 3H), 6.38(s, 1H), 6.66(d, J = 16.1 Hz, 1H), 6.91(d, J = 7.8 Hz, 1H), 7.03(s, 1H), 7.11 (d, J = 8.0 Hz, 1H) 7.31(dd, J = 8.0, 7.8 Hz, 1H), 7.34 (d, J = 16.1 Hz, 1H), 7.57(s, 1H) ppm. ^{13}C NMR (75 MHz, CDCl3) δ 56.7, 55.5, 112.8, 113.5, 115.7, 119.8, 120.4, 130.2, 136.3, 136.5, 137.4, 148.9, 160.2, 161.5, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{15}H_{14}O_4$ 258.2693; found 258.2689.

(E)-5-methoxy-2-(4-methoxystyryl)-4H-pyran-4-one (6c). The synthesis of **6c** was similar to that of **6a**. Yield: 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.78(s, 3H), 3.84(s, 3H), 6.33(s, 1H), 6.53 (d, J = 16.0 Hz, 1H), 6.9(d, J = 8.8 Hz, 2H), 7.31(d, J = 16.0 Hz, 1H), 7.46(d, J = 8.8 Hz, 2H), 7.54(s, 1H) ppm. ^{13}C NMR (75 MHz, CDCl3) δ 55.6, 55.6, 112.7, 114.6, 117.1, 127.9, 129.3, 136.0, 137.2, 148.8, 161.2, 162.1, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{15}H_{14}O_4$ 258.2693; found 258.2693.

(E)-2-(3,4-dimethoxystyryl)-5-methoxy-4H-pyran-4-one (6d). The synthesis of **6d** was similar to that of **6a**. Yield: 72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80(s, 3H), 3.91(s, 3H), 3.93(s, 3H), 6.33(s, 1H), 6.53(d, J = 16.0 Hz, 1H), 6.86(d, J = 8.3 Hz, 1H), 7.03 (s, 1H), 7.07(d, J = 8.3, 1H), 7.30(d, J = 16.0 Hz, 1H), 7.54 (s, 1H) ppm. ^{13}C NMR(75 MHz, $CDCl^3$) δ 56.1, 56.1, 56.6, 109.4, 111.3, 112.7, 11.7, 122.1, 128.1, 136.2, 137.2, 148.8, 149.5, 150.9, 161.9, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{16}H_{16}O_5$ 288.2952; found 288.2948.

(E)-2-(3,5-dimethoxystyryl)-5-methoxy-4H-pyran-4-one (6e). The synthesis of **6e** was similar to that of **6a**. Yield: 79%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.78(s, 3H), 3.81(s, 6H), 6.35(s, 1H), 6.46(s, 1H), 6.63(s, 2H), 6.62(d, J = 16.0 Hz, 1H), 7.27(d, J = 16.0 Hz, 1H), 7.55(s, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl^3$) δ 55.6, 55.6, 76.8, 77.2, 77.6, 102.1, 105.7, 113.5, 119.9, 136.4, 136.9, 137.3, 148.8, 161.3, 161.4 ppm. HRMS $(M+1)^+$ Calcd for $C_{16}H_{16}O_5$ 288.2952; found 288.2951.

(E)-2-(3,4,5-trimethoxystyryl)-5-methoxy-4H-pyran-4-one (6f). The synthesis of **6f** was similar to that of **6a**. Yield: 77%. ^{1}H NMR

(300 MHz, $CDCl_3$) δ 3.8(s, 3H), 3.87(s, 3H), 3.90(s, 6H), 6.36 (s, 1H), 6.57(d, $J = 16.0$ Hz, 1H), 6.72(s, 1H), 7.28(d, $J = 16.0$ Hz, 1H), 7.55(s, 1H) ppm. ^{13}C NMR (75 MHz,$CDCl^3$) δ 56.3, 56.5, 61.1, 104.8, 113.1, 118.6, 130.5, 136.3, 137.2, 139.9, 148.7, 153.6, 161.5, 174.4 ppm. HRMS $(M+1)^+$ Calcd for $C_{17}H_{18}O_6$ 318.3212; found 318.3209.

(E)-2-(2-(benzo[d][1,3]dioxol-5-yl)vinyl)-5-methoxy-4H-pyran-4-one (6g). The synthesis of **6g** was similar to that of **6a**. Yield: 72%. ^1H NMR(300 MHz, $CDCl_3$) δ 3.78(s, 3H), 6.00(s, 2H), 6.33 (s, 1H), 6.49(d, $J = 16.0$ Hz, 1H), 6.81(d, $J = 8.0$ Hz, 1H), 6.98 (d, $J = 8.0$ Hz, 1H), 7.02(s, 1H), 7.26(d, $J = 16.0$ Hz, 1H), 7.54 (s, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl^3$) δ 31.1, 56.6, 101.8, 106.2, 108.8, 112.9, 117.6, 123.8, 129.6, 136.1, 137.3, 148.7, 148.8, 161.8, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{15}H_{12}O_5$ 272.2528; found 272.2523.

(E)-5-methoxy-2-(4-(trifluoromethyl)styryl)-4H-pyran-4-one (6h). The synthesis of **6h** was similar to that of **6a**. Yield: 86%. ^1H NMR (300 MHz, $CDCl_3$) δ 3.80(s, 3H), 6.44(s, 1H), 6.76 (d, $J = 16.1$ Hz, 1H), 7.39(d, $J = 16.1$ Hz, 1H), 7.59–7.70 (m, 5H) ppm. ^{13}C NMR (75 MHz, CDCl3) δ 56.7, 114.2, 121.9, 122.2, 126.1, 126.1, 127.8, 134.6, 137.5, 138.4, 149.0, 160.8, 174.5 ppm. HRMS $(M+1)^+$ Calcd for $C_{15}H_{11}F_3O_3$ 296.2412; found 296.2411.

(E)-5-methoxy-2-(4-(trifluoromethoxy)styryl)-4H-pyran-4-one (6i). The synthesis of **6i** was similar to that of **6a**. Yield: 84%. ^1H NMR (300 MHz, $CDCl_3$) δ 3.79(s, 3H), 6.41(s, 1H), 6.65(d, $J = 16.1$, 1H), 7.24(d, $J = 8.2$ Hz, 2H), 7.34(d, $J = 16.1$ Hz,1H), 7.54(d, $J = 8.2$ Hz, 2H), 7.58(s, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl^3$) δ 56.7, 113.8, 120.5, 121.5, 129.1, 132.3, 133.7, 134.7, 137.5, 149.0. 150.2, 161.2, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{15}H_{11}F_3O_4$ 312.2406; found 312.2406.

(E)-2-(4-(dimethylamino)styryl)-5-methoxy-4H-pyran-4-one (6j). The synthesis of **6j** was similar to that of **6a**. Yield: 75%. ^1H NMR(300 MHz, $CDCl_3$) δ 3.01(s, 6H), 3.77(s, 3H), 6.27(s, 1H), 6.43(d, $J = 16.0$ Hz, 1H), 6.67(d, $J = 8.8$ Hz, 2H), 7.28(d, $J = 16.0$ Hz, 1H), 7.39(d, $J = 8.8$ Hz, 2H), 7.51(s, 1H) ppm. ^{13}C NMR(75 MHz, $CDCl^3$) δ 40.3, 56.5, 111.6, 112.1, 114.1, 122.9, 129.2, 136.8, 137.0, 148.6, 151.5, 162.8, 174.6 ppm. HRMS $(M+1)^+$ Calcd for $C_{16}H_{17}NO_3$ 271.3111; found 271.3108

CELL CULTURE

Cancer cells were purchased from Bioresource Collection and Research Center in Taiwan. Each cell line was maintained in the standard medium and grown as a monolayer in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 g/mL streptomycin. Cultures were maintained at 37°C with 5% CO_2 in a humidified atmosphere.

MTT assay for cell viability. Cells were plated in a 96-well microtiter plates at a density of 5×10^3/well and incubated for 24 h. After that, cells were treated with vehicle alone (control) or compounds (drugs were dissolved in DMSO previously) at the concentrations indicated. Treated cells were further incubated for 48 h. Cell survival is expressed as percentage of control cell growth. The 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, 2 mg/mL) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, which can be measured spectrophotometrically. Tumor cells were incubated in each well with serial dilutions of the tested compounds. After 2 days of incubation (37°C, 5% CO_2 in a humid atmosphere) 100 μL of MTT (2 mg/mL in phosphate buffered saline (PBS)) was added to each well and the plate was incubated for a further 2 h (37°C). The resulting formazan was dissolved in 100 μL DMSO and read at 570 nm. The percentage of growth inhibition was calculated by the following equation: percentage growth inhibition = (1-At/Ac) × 100, where At and Ac represent the absorbance in treated and control cultures, respectively. The drug concentration causing a 50% cell growth inhibition (GI_{50}) was determined by interpolation from dose–response curves. All determinations were carried out in four to six separated experiments.

Statistical analysis. Data are presented as the mean ± standard error of the mean from four to six separated experiments. Statistical analyses were performed using Bonferroni *t*-test method after ANOVA for multigroup comparison and Student's *t*-test method for two-group comparison, $p = 0.05$ was considered significant. Analysis of linear regression (at least five data within 20–80% inhibition) was used to calculate GI_{50} value.

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