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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of kojic acid-derived copper-chelating apoptosis inducing agents

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Abstract Three classes of kojic acid derivatives were synthesized and examined for their antiproliferative activity against HeLa cells. Both **8b** and **11** co-treated with copper ion exhibited synergistic effect on the HeLa cell growth inhibition with GI_{50} values of 11.9 and 7.1 μM, respectively. Flow cytometric analysis of HeLa cells revealed that **11**-Cu co-treatment induced the sub-G1 arrest in a dose-dependent manner, suggesting that the growth-inhibitory effect is attributed to DNA fragmentation. Moreover, western blot of HeLa cells cytosolic extracts displayed the cleavage of the 116-kDa protein poly(ADP-ribose) polymerase and activation of caspase-3 by the reduced level of the 32-kDa proenzyme, indicating that the caspase-dependent apoptotic pathway was involved. We further demonstrated that MAPK pathway regulators such as ERK and p38 were activated in response to **11**-Cu co-treatment, suggesting that the intracellular oxidative stress was dramatically stimulated by the copper ion. Taken together, we have successfully synthesized kojic acid-derived copper-induced apoptotic agents.

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Introduction

Kojic acid (**1**) (Fig. 1) is a metabolic compound produced by several species of fungi, such as *Aspergillus*, *Acetobacter*, and *Penicillium* (Yabuta, 1924). Kojic acid has been added to food as an antioxidant, as a preservative to prevent formation of warm-over flavor in beef, as a food additive for preventing enzymatic discoloration of vegetables, crabs, and shrimps, and as a skin lightening or bleaching agent in cosmetic preparations. Moreover, kojic acid has been demonstrated to exhibit bacteriostatic, anti-inflammatory, insecticidal, antibiotic, cytotoxic, and anti-tumor activities (Blumenthal, 2004; Bentley, 2006). Owing to its versatile heterocyclic skeleton, many kojic acid derivatives have shown to exhibit anticancer (Fickova *et al.*, 2008; Yoo *et al.*, 2010), antifungal, antibacterial (Reddy *et al.*, 2010), antimicrobial, and antiviral (Aytemir *et al.*, 2010) activities.

On the other hand, kojic acid used as a skin lightening agent is mainly attributed to its copper-chelating property for inhibiting tyrosinase activity. Tyrosinase is a copper-containing enzyme responsible for the biosynthesis of the melanin precursor 3,4-dihydroxyphenylalanine (DOPA) (Cabanes *et al.*, 1994). Accordingly, kojic acid has shown to be a bidentate ligand to chelate with copper ion (II) that forms a copper-kojate dimmer as indicated in Fig. 1 (Toy and Smith, 1971).

Accordingly, biological activities of kojic acid derivatives may not depend not only on their physicochemical properties such as hydrophilicity but also on their metal-chelating capability. On the basis of therapeutic indication, copper chelators have revealed to demonstrate anticancer property. For example, tetrathiomolybdate has been shown to suppress

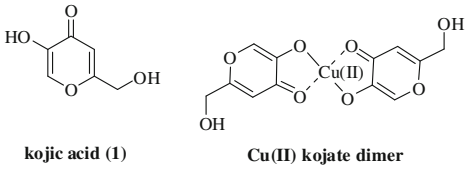


Fig. 1 Chemical structures of kojic acid (1) and Cu(II) kojate dimer

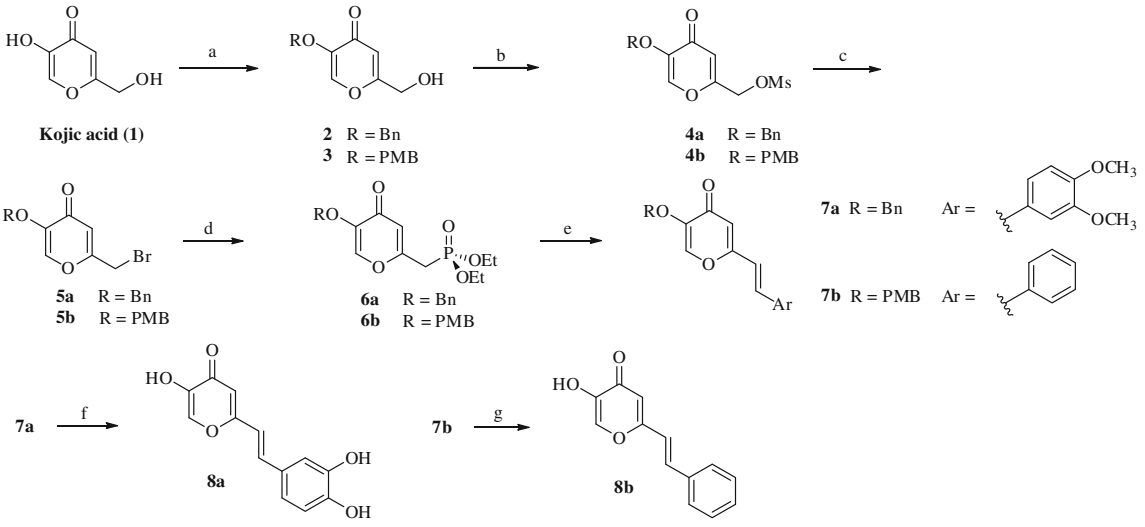
tumor growth, tumor metastases, and angiogenesis and is being investigated in clinical trials (Brewer *et al.*, 2000; Redman *et al.*, 2003). PDTC, a pyrrolidine derivative of dithiocarbamate, is an ionophore capable of transporting copper ion across cell membranes for inhibiting NF-κB signaling pathway (Nobel *et al.*, 1995; Burkitt *et al.*, 1998). In light of its copper-chelating property with tyrosinase, kojic acid appears to be a potential candidate to develop novel classes of metal-chelating antiproliferative agents. To the best of our knowledge, growth inhibition of cancer cells in response to the treatment of kojic acid derivatives in combination of copper ion has not yet been examined. To gain insight into the anticancer activity of kojic acid-derived analogs assisted by copper ion, herein we present the synthesis of several classes of kojic acid derivatives for the evaluation of copper ion co-treated antiproliferative effect on HeLa cells.

Results and discussion

Chemistry

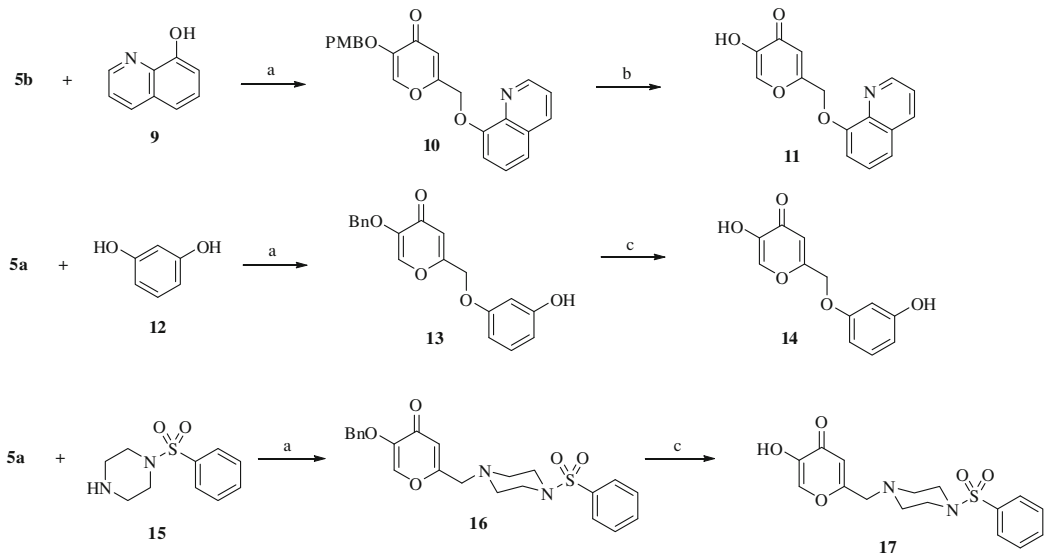
As shown in Schemes 1 and 2, three classes of kojic acid derivatives were synthesized for biological evaluations. As indicated, the C-5 hydroxyl group in kojic acid (**1**) was selectively protected with benzyl (Bn) and *p*-methoxybenzyl (PMB) groups in the presence of potassium carbonate in DMF solution to afford **2** and **3** in 86 and 89 % isolated yields, respectively. The C-7 hydroxyl group of **2** and **3** was subjected to undergo mesylation with methanesulfonyl chloride (MsCl) in the presence of triethylamine in DCM to afford **4a** and **4b** in 92 and 95 % isolated yields, respectively. Both mesylate esters **4a** and **4b** converted to bromide derivatives with sodium bromide in DMF gave **5a** (92 %) and **5b** (87 %), respectively (Imafuku et al., 1979). Arbuzov reaction of **5a** and **5b** with triethylphosphite ($P(OEt)_3$) in toluene at reflux gave phosphonates **6a** and **6b** in 90 and 89 % isolated yields, respectively. To synthesize ethylene-containing derivatives, the Horner-Emmons reaction was employed in which phosphonates **6a** and **6b** were treated with strong base sodium hydride (NaH) in THF followed by the addition of benzaldehydes to obtain **7a** and **7b** in 58 and 64 % isolated yields, respectively. Finally, deprotection of both benzyl and methyl groups in **7a** was carried out by the treatment of boron tribromide (BBr_3) in DCM to give **8a** in 63 % isolated yield. On the contrary, PMB group in **7b** was removed by 10 % trifluoroacetic acid (TFA) in DCM to obtain **8b** (55 %).

Apart from the ethylene group as a linker to generate **8a** and **8b**, C-7 hydroxyl group was modified with an ether linkage in which an aromatic ring was appended. As shown in Scheme 2, bromides **5b** and **5a** were treated with the corresponding 8-hydroxyquinoline **9** and resorcinol **12** in the presence of K_2CO_3 in acetone to obtain **10** and **13** in 78 and 51 % isolated yields, respectively. Next, the removal



Reagents and conditions: a) PMBCl or BnBr, K_2CO_3, DMF; b) MsCl, NEt_3; c) NaBr, DMF; d) $P(OEt)_3$, THF, reflux; e) ArCHO, NaH, THF; f) BBr_3, DCM, -40°C to rt; g) TFA, DCM, rt

Scheme 1 Synthesis of kojic acid-derived **7a**, **7b**, **8a** and **8b**



Reagents: a) K_2CO_3, acetone; b) TFA, DCM, rt; c) BBr_3, DCM, -20 °C to rt.

Scheme 2 Synthesis of kojic acid-derived **11**, **14** and **17**

of *p*-methoxybenzyl group in **10** and benzyl group in **13** were carried out based on the above-mentioned conditions to afford the corresponding products **11** and **14** in yields of 72 and 46 %, respectively. On the other hand, we also introduced the piperazine moiety as a spacer to anchor the kojic acid moiety contributed from **5a** and an arylsulfonylpiperazine **15** to afford **16** (52 %), followed by deprotection of the benzyl group to obtain the product **17** in 86 % yield.

Pharmacological Study

We took advantage of HeLa cells as our working model to examine the antiproliferative effect of kojic acid and its derivatives with and without copper ion treatment. HeLa cells were exposed to the tested compounds at 25 μM in the presence of 10 % FBS DMEM medium for 48 h treatment in the presence and absence of 50 μM copper ion. The antiproliferation of tested compounds was employed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. As shown in Fig. 2, growth inhibition of HeLa cells was not observed by kojic acid treatment with and without co-treated copper ion. This result might be attributed to the poor hydrophobic property of kojic acid for penetrating hydrophobic cell membrane. The above-mentioned result could be in agreement with those of **8a** and **8b**.

As shown, neither **8a** nor **8b** exhibited antiproliferative activity in the absence of copper ion. Nevertheless, **8b** significantly displayed antiproliferative effect in response to copper ion co-treatment with 90 % growth inhibition. Similar to that of kojic acid, **8a** did not show any

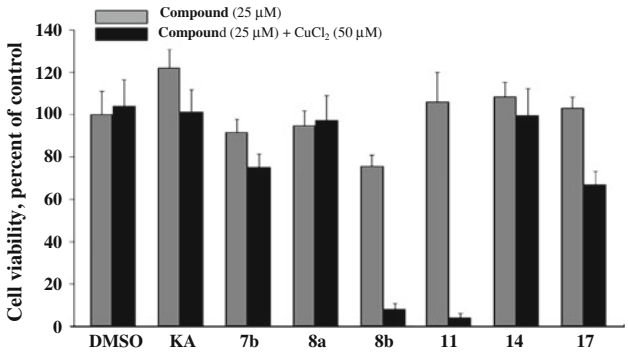


Fig. 2 Antiproliferation of HeLa cells in response to tested compounds (25 μM) in the presence and absence of copper ion (50 μM)

synergistic antiproliferative effect in the presence of copper ion, suggesting that hydrophobicity might be a key factor for antiproliferative activity. Moreover, exposure of HeLa cells to PMB-protected **7b** dramatically abolished the copper-assisted antiproliferative effect in comparison to its counterpart **8b**, indicating that free 5-hydroxyl group along with its adjacent carbonyl group play a pivotal role for copper-chelating capability. Likewise, both **11** and **14** showed no activity in the absence of copper ion. Interestingly, **11** exhibited synergistic effect on HeLa cell growth inhibition in response to copper ion co-treatment with 95 % growth inhibition, while **14** did not show any effect even in the presence of copper ion. Again, this result was similar to that of **8a** with poor hydrophobic property for penetrating cell membrane. However, HeLa cells treated with **17** in the presence of copper ion merely caused 35 % growth inhibition which was not as significant as those of

8b and **11**. Together, our data suggest that potentiation of the antiproliferative effect assisted by copper addition is an indicator of the ionophoric properties of a compound able to raise intracellular copper concentrations to toxic levels. Furthermore, more detailed evaluation of antiproliferative activity indicated that in the presence of 50 μM copper ion, both **8b** and **11** exhibited potential synergistic effect on HeLa cell growth inhibition with GI_{50} values of 11.9 and 7.1 μM, respectively (Table 1).

The mechanistic study of growth inhibition of HeLa cells mediated by **11** in the presence of copper ion was examined by flow cytometric analysis. As shown in Fig. 3, exposure of HeLa cells to kojic acid (**KA**) at 25 μM did not show any sub-G1 phase arrest either in the absence or presence of 50 μM copper ion (Fig. 3c, d).

On the contrary, induction of the sub-G1 phase arrest in HeLa cells was clearly observed in a dose-dependent manner upon treatment of **11** at 5 and 15 μM in the presence of 50 μM copper ion with 11.85 and 48.56 %, respectively (Fig. 3f, h). As a result, the growth-inhibitory effect on HeLa cells mediated by **11** in the presence of copper ion was attributable to the DNA fragmentation and apoptotic cell death.

We further evaluated HeLa cells apoptosis induced by **11** in the presence of copper ion by western blotting assay. As shown in Fig. 4, exposure of HeLa cells to **KA** and **11** at indicated concentration in the absence and presence of copper ion for 24-h treatment showed that induction of poly(ADP-ribose) polymerase (PARP) cleavage by **11** at 15 μM in the presence of 50 μM copper ion was clearly observed. In addition, the disappearance of PARP at 89 kDa was in accordance with the activation of caspase-3, as shown by the reduced level of the 32 kDa proenzyme, suggesting that apoptosis mediated by **11** along with copper ion was, in part, attributed to the caspase-dependent pathway resulting in PARP cleavage and DNA fragmentation. Moreover, the activation of mitogen-activated

Table 1 GI_{50} values of **KA**, **8b** and **11** against HeLa cells

Entry	GI^{a}_{50} (μM)	
	0	50 $CuCl_2$ (μM)
KA	ND	ND
8b	64.5 ± 3.3	11.9 ± 1.9
11	15.2 % inhibition at 75 μM treatment	7.1 ± 2.5

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

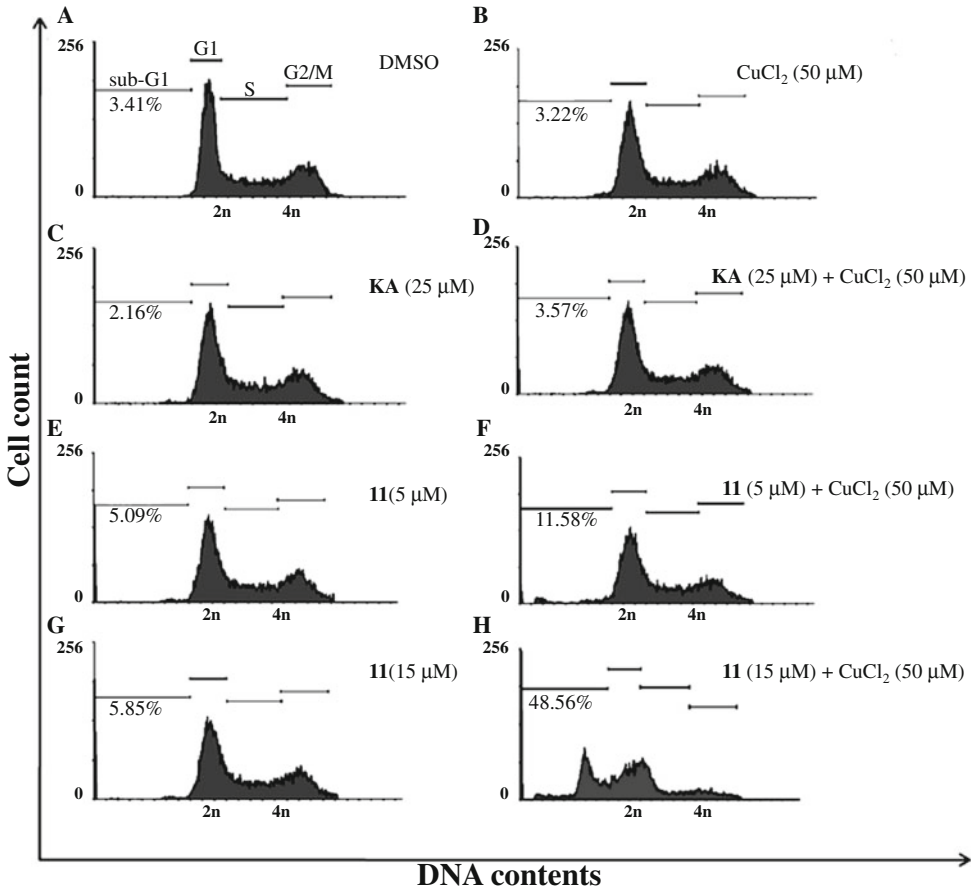


Fig. 3 Flow cytometric analysis of HeLa cells. Cells were harvested after 24-h treatment followed by fixation and propidium iodide staining before the flow cytometric analysis. Sub-G1 phase indicated the DNA fragmentation and cell death. Exposure of HeLa cells to kojic acid (KA) at 25 μM did not enhance sub-G1 phase arrest in the absence and presence of 50 μM copper ion. The sub-G1 phase arrest was observed in a dose-dependent manner upon treatment of **11** at 5 μM and 15 μM in the presence of 50 μM copper ion (11.58 and 48.56 %, respectively). **a** Cells were treated with 0.5 % DMSO as the control; **b** $CuCl_2$ 50 μM; **c** Treatment of KA at 25 μM; **d** Treatment of KA (25 μM) and $CuCl_2$ (50 μM); **e** Treatment of **11** at 5 μM; **f** Treatment of **11** (5 μM) and $CuCl_2$ (50 μM); **g** Treatment of **11** at 15 μM; **h** Treatment of **11** (15 μM) and $CuCl_2$ (50 μM)

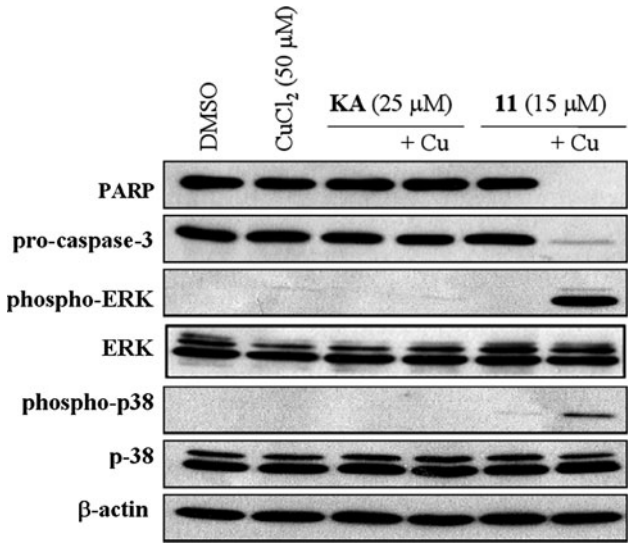


Fig. 4 Induction of apoptosis and activation of MAPK pathway in HeLa cells by **11** in the presence of copper ion after 24-h treatment. The disappearance of 115-kDa poly(ADP-ribose) polymerase (PARP) proteolysis was monitored by western blotting, which was in accordance with the reduced level of the 32 kDa pro-caspase-3. The phosphorylation of ERK and p38 was clearly observed upon co-treatment of **11** and copper ion

protein (MAP) kinases such as ERK and p38 was clearly observed due to the increasing of intracellular oxidative stress mediated by copper ion. As a consequence, kojic acid-derived **11** has demonstrated its metal-chelating capability to shuttle copper ion into the cytosol for MAPK pathway activation and induction of DNA fragmentation and apoptosis.

Conclusions

In summary, in the present study, we synthesized three classes of kojic acid derivatives and examined their growth-inhibitory activity in the absence and presence of copper ion against HeLa cells. We found that **8b** and **11** exhibited dramatically enhanced antiproliferative activity against HeLa cells in the presence of copper ion with GI_{50} values of 11.9 and 7.1 μM, respectively. Flow cytometric analysis of HeLa cells revealed that **11** co-treated with copper ion induced the sub-G1 arrest in a dose-dependent manner, suggesting that the growth-inhibitory effect is attributed to DNA fragmentation. Moreover, western blot of HeLa cells cytosolic extracts prepared from **11** co-treated with copper ion demonstrated the cleavage of 116-kDa protein poly(ADP-ribose) polymerase (PARP), which was in accordance with the activation of caspase-3 by the reduced level of the 32-kDa proenzyme. Taken together, we have successfully synthesized kojic acid derivatives as copper-assisted antiproliferative agents.

Experimental

Chemistry

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

5-(Benzyloxy)-2-(hydroxymethyl)-*4H*-pyran-4-one (**2**)

To a stirred solution of kojic acid (**1**) (2.5 g, 17.6 mmol) and K_2CO_3 (5.3 g, 38.4 mmol) in dry DMF (24 ml) benzyl bromide (3.43 g, 20.09 mmol) was added; the reaction mixture was heated at 50 °C for 2 h. The solvent was removed under reduced pressure and the precipitate was filtered, washed with ethyl acetate and brine, and evaporated in vacuo to obtain crude **2**. The crude product was purified by silica gel chromatography to afford **2** (3.5 g, 86 %) mp 132–133 °C (lit. value 132 °C (Imafuku et al., 1979)). ^{1}H-NMR ($CDCl_3$) δ 4.45 (s, 2H), 5.05 (s, 2H), 6.51 (s, 1H), 7.36 (m, 5H), 7.51 (s, 1H) ppm.

2-(Hydroxymethyl)-5-(4-methoxybenzyloxy)-*4H*-pyran-4-one (**3**)

The synthesis of **3** was similar to that of **2** except for the replacement of BnBr with PMBCl. Yield: 89 %. ^{1}H-NMR (DMSO-d_6) δ 3.75 (s, 3H), 4.29 (s, 2H), 4.86 (s, 2H), 6.31 (s, 1H), 6.84 (d, $J = 8.6$ Hz, 2H), 7.34 (d, $J = 8.6$ Hz, 2H), 8.14 (s, 1H) ppm. ^{13}C-NMR(75 MHz,DMSO) δ 55.5, 60.9, 112.4, 114.2, 127.8, 129.8, 141.8, 147.0, 159.9, 167.5, 158.7, 175.4 ppm.

(5-(Benzyloxy)-4-oxo-*4H*-pyran-2-yl)methyl methanesulfonate (**4a**)

To a stirred solution of **2** (1.16 g, 5 mmol) in dichloromethane (20 ml) methanesulfonyl chloride (0.565 g, 5 mmol) at 0 °C was added, triethylamine (1.02 g, 10 mmol) was added dropwise to the solution. After stirring for 10 min, the mixture was diluted with dichloromethane (20 ml) and washed with brine (40 ml). The organic layer was dried over $MgSO_4$, concentrated in vacuo, and purified by flash column chromatography to afford **4a** (1.42 g, 92 %). ^{1}H-NMR (300 MHz, $CDCl_3$) 3.89 (s, 3H), 4.95 (s, 2H), 5.09 (s, 2H), 6.52 (s, 1H), 7.38–7.39 (m, 5H), 7.57 (s, 1H) ppm. ^{13}C-NMR (75 MHz,

$CDCl_3$) δ 38.2, 56.1, 71.8, 115.5, 127.7, 128.5, 128.73, 135.3, 141.6, 147.4, 158.7, 173.9 ppm.

(5-(4-Methoxybenzyloxy)-4-oxo-*4H*-pyran-2-yl)methyl methanesulfonate (**4b**)

The synthesis of **4b** was similar to that of **4a**. Yield: 95 %. ^{1}H-NMR(300 MHz,DMSO-d_6) δ 3.08(s, 3H), 3.80(s, 3H), 4.94(d, $J = 5$ Hz, 4H), 6.51(s, 1H), 6.87 (d, $J = 3$ Hz, 2H), 7.28(d, $J = 3$ Hz, 2H), 7.56(s, 1H) ppm. ^{13}C-NMR(75 MHz, DMSO-d_6) δ 38.4, 55.5, 65.3, 71.9, 114.3, 115.8, 127.6, 129.8, 142.0, 147.5, 158.8, 160.4, 174.2 ppm.

5-(Benzyloxy)-2-(bromomethyl)-*4H*-pyran-4-one (**5a**)

To a stirred solution of **4a** (0.93 g, 3 mmol) in DMF (15 ml) NaBr (0.61 g, 6 mmol) was added, the mixture was stirred at room temperature for 20 min. After filtering the excess amount of NaBr, the filtrate was diluted with ethyl acetate (50 ml) and washed with water (25 ml) and brine (40 ml). The organic layer was dried over $MgSO_4$, concentrated in vacuo, and purified by silica gel chromatography (ethyl acetate/dichloromethane = 3/7) to obtain **5a**. Yield: 92 %. ^{1}H-NMR(300 MHz,$CDCl_3$) δ 4.14(s, 2H), 5.08(s, 2H), 6.46(s, 1H), 7.35–7.40(m, 5H), 7.56(s, 1H) ppm. ^{13}C-NMR(75 MHz,$CDCl_3$) δ 26.4, 72.0, 115.1, 127.9, 128.6, 128.8, 135.6, 141.7, 147.4, 161.7, 174.4 ppm.

5-(4-Methoxybenzyloxy)-2-bromomethyl-*4H*-pyran-4-one (**5b**)

The synthesis of **5b** was similar to that of **5a**. Yield: 87 %. ^{1}H-NMR(300 MHz, $CDCl_3$) δ 3.79(s, 3H), 4.13(s, 2H), 4.99(s, 2H), 6.44(s, 1H), 6.88(d, $J = 6.7$ Hz, 2H), 7.30(d, $J = 6.7$ Hz, 2H), 7.54(s, 1H) ppm. ^{13}C-NMR(75 MHz, DMSO-d_6) δ 38.4, 55.5, 71.9, 114.1, 115.8, 127.2, 129.8, 142.1, 147.4, 158.7, 160.3, 174.3 ppm.

Diethyl (5-(benzyloxy)-4-oxo-*4H*-pyran-2-yl)methylphosphonate(**6a**)

To a solution of **5a** (1.47 g, 5 mmol) in toluene (10 ml) triethylphosphite (1.66 g, 10 mmol) was added and the mixture was stirred at reflux for 24 h. The solvent and excess triethylphosphite were removed in vacuo and the resulting oily residue was purified by flash column chromatography (ethyl acetate/dichloromethane = 4/1) to afford **6a**. Yield: 86 %. ^{1}H-NMR(300 MHz,$CDCl_3$) δ 1.28(t, $J = 7.1$ Hz, 6H), 2.98(s, 1H), 3.05(s, 1H), 4.09(m, 4H), 5.04(s, 2H), 6.34(s, 1H), 7.27–7.38(m, 5H), 7.50(s, 1H) ppm. ^{13}C-NMR(75 MHz,$CDCl_3$) δ 16.2, 31.3, 62.7, 71.7, 115.8, 127.7, 128.3, 128.6, 135.6, 141.6, 146.9, 159.3, 174.1 ppm.

Diethyl ((5-((4-methoxybenzyl)oxy)-4-oxo-*4H*-pyran-2-yl)methyl)phosphonate (**6b**)

The synthesis of **6b** was similar to that of **6a**. Yield: 95 %. ^{1}H-NMR(300 MHz,$CDCl_3$) δ 1.28(t, $J = 7.1$ Hz, 6H), 2.98(s, 1H), 3.05(s, 1H), 3.78(s, 3H), 4.11(m, 4H), 5.03(s, 2H), 6.34(s, 1H), 6.89(d, $J = 6.7$ Hz, 2H), 7.32(d, $J = 6.7$ Hz, 2H), 7.57(s, 1H) ppm. δ 16.2, 31.3, 38.4, 55.6, 71.9, 114.1, 115.8, 127.2, 129.7, 142.1, 147.3, 158.7, 160.3, 174.3 ppm.

(E)-5-(Benzyloxy)-2-(3,4-dimethoxystyryl)-*4H*-pyran-4-one (**7a**)

To a stirred solution of **5** (0.352 g, 1 mmol) and NaH (0.08 g, 2 mmol, 60 % suspension in mineral oil) in dry THF (20 ml) a solution of 3,4-dimethoxybenzaldehyde (0.166 g, 1 mmol) in THF (5 ml) was added dropwise under Ar 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** (0.211 g, 58 %). ^{1}H NMR(300 MHz, $CDCl_3$) δ 3.9 (s, 6H), 5.11 (s, 2H), 6.35 (s, 1H), 6.52 (d, $J = 16.1$ Hz, 1H), 6.87 (d, $J = 8.2$ Hz, 1H), 7.02 (s, 1H), 7.07(d, $J = 8.2$ Hz, 1H), 7.27(d, $J = 16.1$ Hz, 1H), 7.33–7.44 (m, 5H), 7.52 (s, 1H) ppm. ^{13}C NMR(75 MHz, $CDCl_3$) δ 56.1, 72.1, 109.4, 111.3, 113.3, 117.1, 122.01, 127.9, 128.4, 128.7, 136.2, 141.3, 147.0, 49.5, 150.9, 161.7, 175.1, 191.9 ppm.

(E)-5-(Benzyloxy)-2-styryl-*4H*-pyran-4-one (**7b**)

Compound **7b** was synthesized from the procedure described for compound **7a**. Yield (64 %). ^{1}H-NMR(300 MHz,$CDCl_3$) δ 5.11(s, 2H), 6.40 (s, 1H), 6.65 (d, $J = 16.1$ Hz, 1H), 7.32 (d, $J = 16.1$ Hz, 1H), 7.34–7.50 (m, 10H), 7.51(s, 1H) ppm. ^{13}C NMR(75 MHz,$CDCl_3$) δ 72.2, 114.0, 119.4, 127.7, 128.0, 128.6, 128.9, 129.1, 129.9, 135.0, 136.1, 136.4, 141.4, 147.2, 161.4, 175.2 ppm.

(E)-2-(3,4-Dihydroxystyryl)-5-hydroxy-*4H*-pyran-4-one (**8a**)

To a solution of **6a** (0.364 g, 1.0 mmol) in DCM (20 ml) boron tribromide (1.5 g, 6 mmol) at −20 °C was added; the mixture was allowed to room temperature and stirred for 24 h. The reaction mixture was diluted with methanol (10 ml), washed with water (40 ml) and brine (40 ml). The organic layer was dried over $MgSO_4$, evaporated in vacuo, and purified by purified by flash column chromatography (ethyl acetate: methanol = 4/1) to afford **6a** (0.155 g,

63 %). ^{1}H-NMR(300 MHz, DMSO-d_6) δ 6.43 (s, 1H), 6.73 (d, $J = 16.1$ Hz, 1H), 6.75 (d, $J = 8.1$ Hz, 1H), 6.92 (d, $J = 8.1$, 1H), 7.02 (s, 1H), 7.20 (d, $J = 16.1$ Hz, 1H), 8.00 (s, 1H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 110.9, 114.3, 115.9, 116.4, 120.6, 126.7, 135.7, 138.9, 145.7, 147.6, 161.9, 163.1, 174.1 ppm. HRMS $(M + 1)^+$ calcd for $C_{13}H_{11}O_5$ 247.0606, found 247.0607.

(E)-5-Hydroxy-2-styryl-*4H*-pyran-4-one (**8b**)

To a solution of **7b** (0.304 g, 1 mmol) in 10 % trifluoroacetic acid in DCM (5 ml), the resulting mixture was stirred at room temperature for 30 min. The solvent was removed and the residue was purified by flash silica gel chromatography (DCM ethyl acetate = 1/1) to give **8b** with 55 % yield. ^{1}H-NMR (300 MHz, MeOH-d_4) δ 6.50 (s, 1H), 6.96 (d, $J = 16.2$ Hz, 1H), 7.35 (m, 1H), 7.40 (d, $J = 7.8$ Hz, 2H), 7.47 (d, $J = 16.2$ Hz, 1H), 7.61 (d, $J = 7.8$ Hz, 1H) ppm. ^{13}C-NMR (75 MHz, MeOH-d_6) δ 102.8, 110.9, 119.0, 119.2, 120.0, 120.5, 121.2, 127.0, 128.1, 131.1, 154.6 ppm. HRMS $(M + 1)^+$ calcd for $C_{13}H_{11}O_3$ 215.0708, found 215.0713.

5-(4-Methoxybenzyloxy)-2-((quinolin-8-yloxy)methyl)-*4H*-pyran-4-one (**10**)

To a solution of **5b** (0.325 g, 1 mmol) in dry acetone (7 ml) K_2CO_3 (0.256 g, 2 mmol) and 8-hydroxyquinoline **9** (0.145 g, 1 mmol) was added; the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was filtered to remove K_2CO_3. The filtrate was evaporated in vacuo to obtain the crude product. The crude product was purified by flash chromatography (hexane: ethyl acetate = 3/1) to afford **10**. Yield: 78 %. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.80(s, 3H), 5.00(s, 2H), 5.18(s, 2H), 6.62(s, 1H), 6.87(d, $J = 9$ Hz, 2H), 7.05(s, 1H), 7.30(d, $J = 6$ Hz, 2H), 7.42(m, 3H), 7.57(s, 1H), 8.15(s, 1H), 8.95(s, 1H) ppm.

5-Hydroxy-2-((quinolin-8-yloxy)methyl)-*4H*-pyran-4-one (**11**)

To a solution of **10** (0.195 g, 0.5 mmol) in 10 % trifluoroacetic acid in DCM (5 ml), the resulting mixture was stirred at room temperature for 30 min. The solvent was removed and the residue was purified by flash silica gel chromatography (DCM ethyl acetate = 1/1) to give **11** with 72 % yield. ^{1}H-NMR (300 MHz, MeOH-d_4) δ 5.35(s, 2H), 6.72(m, 1H), 7.15(m, 1H), 7.62(m, 1H), 7.78(m, 2H), 7.83(m, 2H), 8.94(d, $J = 9$ Hz, 1H), 9.03(d, $J = 6$ Hz, 1H) ppm. HRMS $(M + 1)^+$ calcd for $C_{15}H_{12}NO_4$ 270.0766, found 270.0758.

5-(Benzyloxy)-2-((3-hydroxyphenoxy)methyl)-*4H*-pyran-4-one (**13**)

The synthesis of **13** was similar to that of **10** except for the replacement of **5b** and **9** with **5a** and resorcinol **12**, respectively. Yield: 51 % yield. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 4.93(s, 4H), 6.38(m, 3H), 6.49(s, 1H), 7.06(m, 1H), 7.39(s, 5H), 8.25(s, 1H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 65.0, 70.5, 102.1, 105.3, 108.8, 113.6, 128.1, 128.2, 128.4, 130.0, 136.0, 141.5, 146.9, 158.6, 158.6, 162.7, 172.9 ppm.

5-Hydroxy-2-((3-hydroxyphenoxy)methyl)-*4H*-pyran-4-one (**14**)

The synthesis of **14** was similar to that of **8a** with 46 % yield. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 4.92(s, 2H), 6.38(m, 4H), 7.03(t, $J = 9$ Hz, 1H), 8.09(s, 1H), 9.20(s, 1H), 9.47(s, 1H) ppm. HRMS $(M + 1)^+$ calcd for $C_{12}H_{11}O_5$ 235.0606, found 235.0604.

5-(Benzyloxy)-2-((4-(phenylsulfonyl)piperazin-1-yl)methyl)-*4H*-pyran-4-one (**16**)

The synthesis of **16** was similar to that of **13** with 52 % yield. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.57(m, 4H), 3.04(s, 4H), 3.33(s, 2H), 5.04(s, 2H), 6.36(s, 1H), 7.35(m, 5H), 7.48(s, 1H), 7.60(m, 3H), 7.52(d, $J = 5$ Hz, 2H) ppm.^{13}C-NMR (75 MHz,DMSO-d_6) δ 46.1, 52.3, 58.8, 72.0, 114.87, 127.9, 127.9, 128.5, 128.8, 129.3, 133.2, 135.4, 135.8, 141.7, 147.2, 163.9, 174.6 ppm.

5-Hydroxy-2-((4-(phenylsulfonyl)piperazin-1-yl)methyl)-*4H*-pyran-4-one (**17**)

The synthesis of **17** was similar to that of **8a** with 86 % yield.^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.15(m, 4H), 3.62(m, 4H), 4.22(s, 2H), 6.55(s, 1H), 7.67(m, 5H), 8.09(s, 1H) ppm. ^{13}C-NMR (75 MHz,DMSO-d_6) δ 43.2, 50.5, 54.9, 55.2, 117.2, 127.7, 129.7, 133.8, 140.5, 146.4, 156.3, 173.6 ppm. HRMS $(M + 1)^+$ calcd for $C_{16}H_{19}N_2O_5S$ 351.1015, found 351.1017.

Cell culture

Cancer cells were purchased from the 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 (DMEM) 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 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 were 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 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 = $(1-\mathrm{At/Ac}) \times 100$, 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 three separated experiments.

Determination of apoptosis by flow cytometry

Apoptosis and cell cycle profile were assessed by DNA fluorescence flow cytometry. HeLa cells treated with DMSO or $CuCl_2$, KA, and **11** at indicated concentrations for 24 h were harvested, rinsed in PBS, re-suspended and fixed in 80 % ethanol, and stored at −20 °C in fixation buffer until it is ready for analysis. Then, the pellets were suspended in 1 ml of fluorochromic solution (0.08 mg/ml PI (propidium iodide), 0.1 % TritonX-100 and 0.2 mg/ml RNase A in 1X PBS) at room temperature in the dark for 30 min. The DNA content was analyzed by FACScan flow cytometer (Becton–Dickinson, Mountain View, CA) and CellQuest software (Becton–Dickinson). The population of apoptotic nuclei (subdiploid DNA peak in the DNA fluorescence histogram) was expressed as the percentage in the entire population.

Protein extraction and western blotting

After the treatment of HeLa cells with vehicle (0.5 % DMSO) or tested compounds for indicated time treatment, the cells were washed twice with PBS and reaction was terminated by the addition of 100 µl lysis buffer. For Western blot analysis, the amount of proteins (50 µg) were separated by electrophoresis in a 15 % SDS–PAGE and transferred to a nitrocellulose membrane. After an overnight incubation at 4 °C in TBST/5 % non-fat milk, the membrane was washed with TBST three times and immunoreacted with the monocolonal primary antibodies, anti-poly-ADP-ribose polymerase(PARP) (1:500), anti-pro-caspase-3 (1:1000), anti-phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti-phospho-p38 (1:1000), anti-p38 (1:1000), and anti-β-actin (1:1000) from Cell Signaling Technology (Beverly, MA). After four washings with TBST, the anti-mouse or anti-rabbit IgG (dilute 1:10,000) was applied to the membranes for 1 h at room temperature. The membranes were washed with TBST for 1 h and the detection of signal was performed using an enhanced chemiluminescence (ECL) detection reagents.

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