Supplement

Tangeretin Sensitizes Cisplatin-resistant Human Ovarian Cancer Cells through Down-regulation of PI3K/Akt Signaling Pathway

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Cell growth and cytotoxicity assay. Cellular proliferation was assessed by the ability of live cells to uptake and convert soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into formazan crystals. Exponentially growing cells were seeded in 96-well plates at an initial density of 5 $\times 10^3$ per well, treated with different concentration of cisplatin or tangeretin and maintained in culture for 72 hours. A stock solution of tangeretin (100 mM) was made in DMSO and appropriate working concentrations were prepared in cell culture medium immediately before use. An aqueous solution of cisplatin was freshly made before the start of each experiment.

Colony formation assay for long-term cell survival. The A2780/CP70 cells were trypsinized and plated in fresh culture medium at a density of 10^3 cells/100 mm plate. Cells were treated with 3 or 6 μM cisplatin or vehicle 24 hours after treatment with 150 μM tangeretin or vehicle. The cells were grown for 10 days and the cell colonies were fixed with 70% ethanol and stained with crystal violet (0.5% in ethanol). The plates were rinsed with water, air-dried, photographed and evaluated for colony estimation.

Results

Cisplatin and tangeretin treatments cause a dose-dependent cell growth inhibition. The anti-proliferative effect of cisplatin and tangeretin was examined in human ovarian cancer cell lines by the MTT assay (Figure S2). 72 hours continuous exposure of A2780, A2780/CP70 and 2008/C13 cells to cisplatin resulted in a dose-dependent cell growth inhibition. The IC_{50} of the A2780, A2780/CP70, 2008 and 2008/C13 cells was 4.4 μM, 19.4, μM, 3.9 μM and 19.8, μM respectively, confirming that

A2780/CP70 and 2008/C13 were about five times more resistant to cisplatin than the parental cells. Tangeretin showed a dose-dependent cell growth inhibition on A2780/CP70 and 2008/C13 cell lines with an IC_{50} of 239.7 and 238.3 μM, respectively. Comparing the IC_{50} of A2780/CP70 and 2008/C13 cell lines with that of other previously tested cancer cell lines, A2780/CP70 and 2008/C13 cells appeared to be highly resistant to tangeretin inhibition (1, 2).

Tangeretin potentiates cisplatin-induced growth inhibition. The sensitizing effects of the pretreatment of A2780/CP70 cells with tangeretin followed by cisplatin was further confirmed using colony forming assay (Figure S3). The colony formation of singly exposed cultures to 150 μM tangeretin was indistinguishable from the untreated controls, while the 3 and 6 μM cisplatin treatments showed a small but dose dependent decrease in colony formation. On the other hand, combined exposures, with tangeretin prior to cisplatin, caused a drastic reduction in the colony formation owing to the severe synergistic effects on the clonogenic survival of cisplatin-resistant A2780/CP70 cells.

Figure Legends

Figure S1. Structure of tangeretin.

Figure S2. Cisplatin and tangeretin exhibit a dose-dependent cell growth inhibitory effect on human ovarian cancer cell lines. *A*, A2780, A2780/CP70, 2008 and 2008/C13 cells were treated with different concentration of cisplatin for 72 hours, cell growth was determined using MTT assay. *B*, A2780/CP70 and 2008/C13cells were treated with tangeretin similar to that with cisplatin. IC50 for each drug was calculated to indicate a dose which achieves 50% cell growth inhibition on corresponding cell line.

Figure S3. Tangeretin potentiates cisplatin-induced growth inhibition. Cells were treated with vehicle, indicated drug or drug combination, in which cells were treated with tangeretin for 24 hours followed by cisplatin treatment for additional 24 hours. The drug treated cells were cultured for 10 days and colonies were fixed with ethanol, stained with crystal violet and photographed for the assessment of colony formation.

References

1 Pan, MH, Chen, WJ, Lin-Shiau, SY, Ho, CT and Lin, JK. Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells, Carcinogenesis 2002;23:1677-1684.

2 Morley, KL, Ferguson, PJ and Koropatnick, J. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells, Cancer Lett 2007;251:168-178.

Table S1. Effect of combining tangeretin and cisplatin in killing resistant cancer cells.

	Schedule	Treatment: Cisplatin (μM)	Treatment: Tangeretin (μM)	Cisplatin/Tangeretin (Ratio)	Combination Index (CI)
A2780/CP70	Tangeretin (24 h)	1.5	25.0	1:16.67	0.712
	+	2.0	50.0	1:25.00	0.775
	Cisplatin	3.0	100.0	1:33.33	0.707
		3.0	150.0	1:50.00	0.695
		6.0	150.0	1:25.00	0.658
	Cisplatin (24 h)	1.5	25.0	1:16.67	1.035
	+	2.0	50.0	1:25.00	1.065
	Tangeretin	3.0	100.0	1:33.33	1.031
		3.0	150.0	1:50.00	1.115
		6.0	150.0	1:25.00	1.028
2008/C13	Tangeretin (24 h)	1.5	25.0	1:16.67	0.739
	+	2.0	50.0	1:25.00	0.696
	Cisplatin	3.0	100.0	1:33.33	0.691
		3.0	150.0	1:50.00	0.680
		6.0	150.0	1:25.00	0.599
	Cisplatin (24 h)	1.5	25.0	1:16.67	1.044
	+	2.0	50.0	1:25.00	0.998
	Tangeretin	3.0	100.0	1:33.33	1.052
		3.0	150.0	1:50.00	1.081
		6.0	150.0	1:25.00	1.033

Cell killing, for dual tangeretin and cisplatin treatment schedules, in A2780/CP70 and 2008/C13 human ovarian cancer cell line was determined as described in Methods. CI, a quantitative measure of the degree of drug interaction, is calculated using CalcuSyn software. The CI <1 indicates synergy; CI =1 indicates additive effects; CI >1 indicates antagonism.

Fig. S1

5,6,7,8,40-pentamethoxyflavone

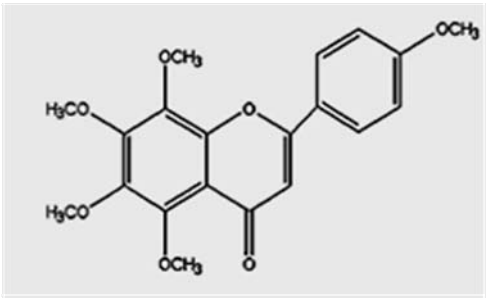


Fig. S2

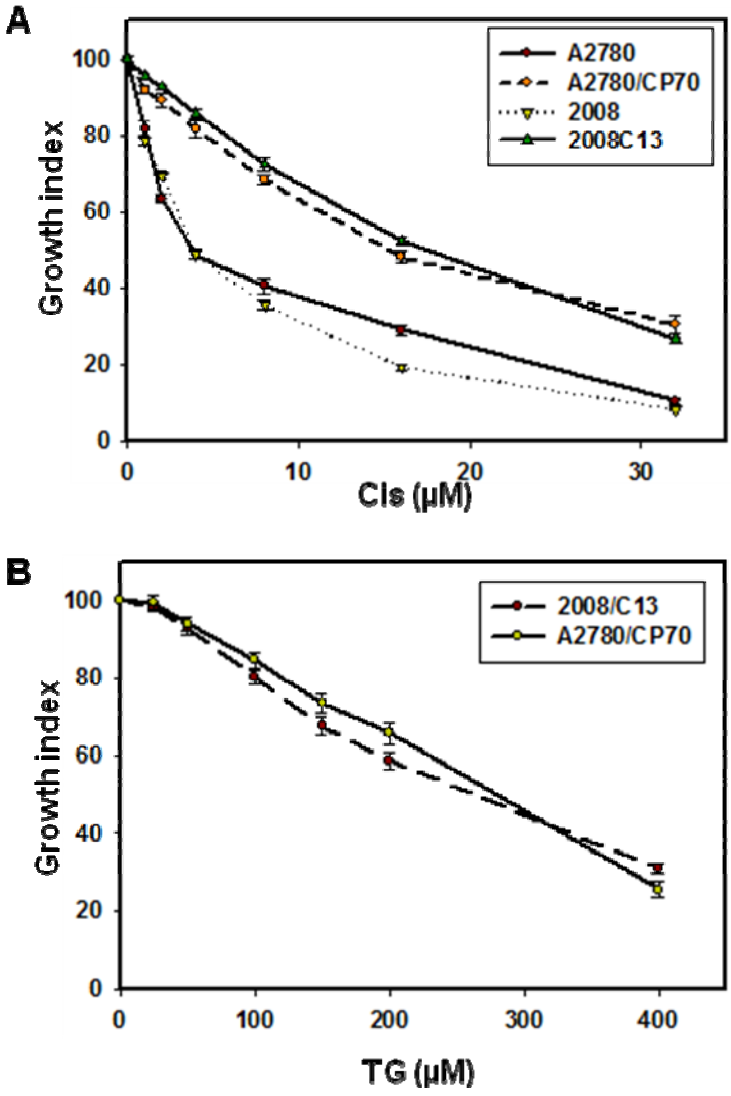
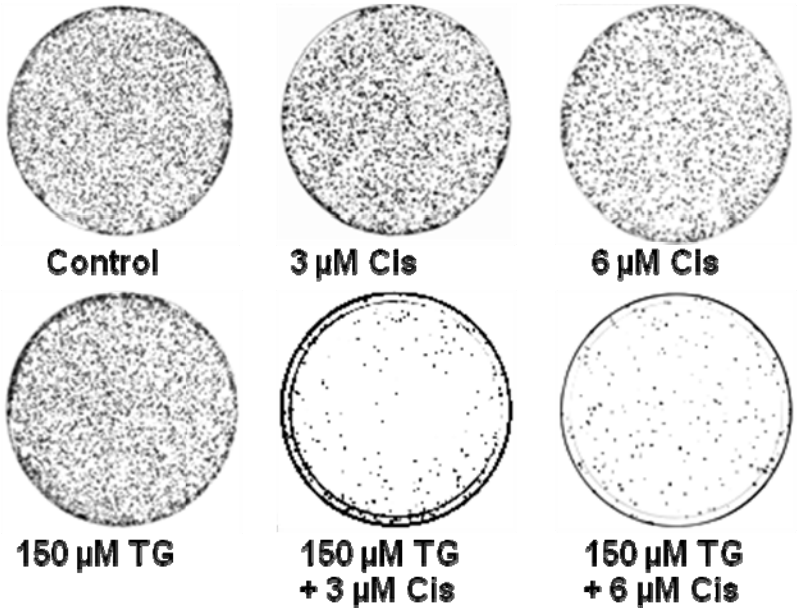


Fig. S3


Control
3 µM Cis
6 µM Cis
150 µM TG
150 µM TG
+ 3 µM Cis
150 µM TG
+ 6 µM Cis