**Tangeretin Sensitizes Cisplatin-Resistant Human Ovarian Cancer Cells through Downregulation of Phosphoinositide 3-Kinase/Akt Signaling Pathway**

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**Abstract**

Combination of innocuous dietary components with anticancer drugs is an emerging new strategy for cancer chemotherapy to increase antitumor responses. Tangeretin is a citrus flavonoid known to inhibit cancer cell proliferation. Here, we show an enhanced response of A2780/CP70 and 2008/C13 cisplatin-resistant human ovarian cancer cells to various combination treatments of cisplatin and tangeretin. Pretreatment of cells with tangeretin before cisplatin treatment synergistically inhibited cancer cell proliferation. This combination was effective in activating apoptosis via caspase cascade as well as arresting cell cycle at G2-M phase. Moreover, phospho-Akt and its downstream substrates, e.g., NF-κB, phospho-GSK-3β, and phospho-BAD, were downregulated upon tangeretin-cisplatin treatment. The tangeretin-cisplatin–induced apoptosis in A2780/CP70 cells was increased by phosphoinositide-3 kinase (PI3K) inhibition and siRNA-mediated Akt silencing, but reduced by overexpression of constitutively activated Akt and GSK-3β inhibition. The overall results indicated that tangeretin exposure preconditions cisplatin-resistant human ovarian cancer cells for a conventional response to low-dose cisplatin-induced cell death occurring through downregulation of PI3K/Akt signaling pathway. Thus, effectiveness of tangeretin combinations, as a promising modality in the treatment of resistant cancers, warrants systematic clinical studies. [Cancer Res 2009;69(23):8910–7]

**Introduction**

Majority of patients with ovarian cancer are not effectively treated with standard cisplatin (cis-diaminedichloroplatinum (II)) regimens primarily due to the obstacle posed by development of drug resistance (1). Like most malignancies, intrinsic drug resistance of ovarian cancers could be attributed to multiple genetic and/or epigenetic alterations resulting in the loss of tumor suppressor functions or dysregulation of survival signals (2). Identification and reversal of these traits offer valuable targets for modulating the response of cancer cells. Development of new effective strategies based on transient targeted response modulation is now in the forefront of cancer research to overcome drug resistance in cancer chemotherapy.

Among the current chemotherapy drug regimens, cisplatin represents one of the clinically most important antineoplastic agent with anticancer activity against a wide variety of solid tumors (3). The antineoplastic effect of cisplatin is mediated by the formation of functionally lethal intrastrand DNA cross-links. These lesions activate damage response pathways that result in diverse effects including DNA synthesis inhibition, RNA transcription suppression, cell cycle arrest, and apoptosis. Several mechanisms are responsible for cisplatin resistance. These include reduced platinum accumulation, enhanced platinum detoxification and metabolism, altered DNA damage repair and, more importantly, the activation of phospholipid kinase, phosphatidylinositol 3-kinase (PI3K)/Akt, and other cellular survival signaling pathways ultimately causing dysregulation of apoptotic pathway (4).

PI3K/Akt signal transduction plays a critical role in the control of cell growth and proliferation (5). The increased Akt activation or dysregulation due to elevated Akt expression and indirect changes in Akt regulators results in stronger cell survival signaling, which is a common feature in various forms of human cancers, including human ovarian carcinoma (6, 7). Dozens of downstream substrates of Akt kinase have been identified including those related to chemotherapy resistance in cancer cells, e.g., GSK-3β, BAD, and transcription factor NF-κB, etc. These substrates directly or indirectly regulate apoptosis. GSK-3β, for example, is phosphorylated by Akt, and GSK-3β itself is involved in the regulation of cell proliferation, antiapoptotic pathways, and cell cycle progression (8–10). BAD, a proapoptotic Bcl-2 family member (11), is an Akt target directly implicated in regulating cell survival (12). Phosphorylation of BAD changes its affinity to Bcl-2 molecules and phospho-BAD (p-BAD) is unable to inhibit Bcl-2 function (13, 14). Akt also regulates NF-κB pathway via phosphorylation and activation of inhibitory κ-B kinase and RelA (15, 16). The NF-κB transcription factors themselves regulate several important physiologic processes, e.g., inflammation and immune responses, cell growth, and apoptosis. Thus, inhibition of NF-κB activation offers a potential strategy for treatment of different malignancies (17, 18).

Newer approaches using dietary flavonoids in combination therapies are being increasingly explored to achieve greater efficacy for drug-resistant cancer cells. For instance, soy isoflavone genistein has been shown to increase apoptosis induced by chemotherapeutic agents in human cancer cells through inactivation of NF-κB via downregulating Akt pathway (19). Flavonoids alone have also been shown to induce apoptosis in some cancer cells, while sparing normal cells (20). Several mechanisms have been suggested for flavonoid-induced apoptosis, including inhibition of topoisomerase I/II...
activity (21–24), regulation of expression of heat shock (25) and Bcl-2 family proteins (26–29), activation of caspase-9 and caspase-3 (22), and modulation of Akt signaling and NF-κB activation (30).

Tangeretin (Supplementary Fig. S1), a polymethoxylated citrus flavonoid, exhibits antiproliferative, anti-invasive, antimitastic, and antioxidant activities (20, 31). The molecular mechanisms and potential applications of tangeretin and other citrus flavonoids in therapy have been reviewed (20, 31, 32). In this study, we tested the potential of tangeretin to sensitize cisplatin-resistant human ovarian cancer A2780/CP70 and 2008/C13 cells to cisplatin-induced cell death using different combination schedules and identified the underlying mechanism of its action. The results showed that tangeretin pretreatment synergizes the low-dose cisplatin-induced cancer cell death. The synergistic effect is mediated through the downregulation of PI3K/Akt pathway. Based on these observations, tangeretin-cisplatin combination may offer a promising new approach in effective treatment of human ovarian cancers.

Materials and Methods

Cell culture and reagents. Human ovarian cancer cells A2780 and its cognate cisplatin-resistant A2780/CP70 were provided by Dr. Paul Modrich (Duke University, Durham, NC). Another pair of cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines 2008 and 2008/C13 was provided by Drs. Francois Claret and Qingxiu Zhang (M.D. Anderson Cancer Center, Houston, TX). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 atmosphere.

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Cell growth and MTT cytotoxicity assay. Cells were seeded in 96-well plates, treated with different concentration of cisplatin or tangeretin, and maintained in culture for 72 h. For the combination experiments, A2780/CP70 and 2008/C13 cells were treated with tangeretin (150 μmol/L) or cisplatin (3 or 6 μmol/L) treatment. Drug-treated cells were incubated for 72 h and then MTT was added to the cultures for an additional 2 h. The medium was replaced with acidified isopropanol for 1 h and the absorbance was determined by Spectramax M5 microplate reader (Molecular Devices). Drug interactions and isobologram were analyzed using CalcuSyn software (Biosoft).

DNA fragmentation analysis for detecting apoptosis. Cells were treated with 150 μmol/L tangeretin or vehicle for 24 h and then exposed to 3 or 6 μmol/L cisplatin or vehicle for another 72 h. Adherent and floating cells were recovered and DNA was isolated and evaluated for fragmentation as described earlier (33).

Quantitative measurement of apoptosis by Histone/DNA ELISA. Cell death ELISA Plus kit was used for detection of apoptosis according to manufacturer’s protocol. Briefly, cells were treated with tangeretin and cisplatin as described above. The cytoplasmic histone/DNA fragments in cell lysates were bound to the immobilized biotin-conjugated histone antibodies in a 96-well streptavidin-coated plate. Wells were exposed to peroxidase-conjugated DNA antibody followed by peroxidase substrate, and the absorbance at 405 nm was measured colorimetrically using Spectramax M5 microplate reader.

Western blot analysis. Cells were treated with tangeretin, cisplatin, or tangeretin-cisplatin combination. The cells were harvested and boiled in...
alysis buffer containing protease inhibitors. Samples were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were incubated with the primary antibodies at 4°C overnight, washed with TBST buffer, and incubated again with an appropriate HRP-conjugated secondary antibody at 37°C for 1 h. The membranes were washed and examined by chemiluminescence detection.

Flow cytometric analysis of cell cycle and apoptosis. Tangeretin and/or cisplatin-treated cells were collected and fixed with 70% ice-cold ethanol overnight at −20°C. Cells were centrifuged, resuspended in a mix of propidium iodide and RNase A, and incubated at 37°C for 40 min. Cells were pelleted, washed, and resuspended in PBS to a final concentration of 1 × 10^6/mL and analyzed using BD FACS Calibur (BD Biosciences).

Transfection of constitutively active Akt plasmid. Cells were seeded in an antibiotic-free medium at 37°C for 24 h and transfected with constitutively active Akt plasmid 14751 or empty vector DNA using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions. After 24 h of transfection, cells were treated as described above and analyzed for cellular proteins and apoptosis.

Akt siRNA transfection. The Akt siRNA kit was used to silence Akt expression according to the manufacturer’s protocol. Briefly, cells were grown for 24 h to ~50% confluency and medium was replaced with 500 μL of an antibiotic-free medium. siRNA-transfection reagent mixture was applied to cultured cells for 48 h. Cells were then treated as described above and analyzed for cellular proteins and apoptosis.

Results

Tangeretin potentiates cisplatin-induced growth inhibition in a drug sequence–dependent manner. First, the antiproliferative effect of cisplatin and tangeretin was examined in cisplatin-resistant A2780/CP70 and 2008/C13 human ovarian cancer cell lines (Supplementary Fig. S2). Next, we tested whether tangeretin can sensitize cells to low-dose cisplatin-induced death. Cells were exposed to varying concentrations of tangeretin (25–150 μmol/L) and cisplatin (1.5–6 μmol/L) under two different drug administration scenarios: an initial 24-hour tangeretin exposure followed by 72-hours cisplatin treatment and vice versa. The cell growth was determined and the values of combination index (CI), a quantitative measure of drug interaction, were calculated (Supplementary Table S1; Fig. 1). As shown in Fig. 1, pretreatment of cells with tangeretin for 24 hours synergized the cytotoxic effect of cisplatin. The CI ranged from approximately 0.5 to 0.7 for every tangeretin–cisplatin combination tested. On the other hand, when cisplatin was administered before tangeretin, CI was between 1 and 1.1, indicating additive rather than synergistic effects. We selected two cisplatin doses (3 and 6 μmol/L) that kill about 20% to 30% of resistant cells and combined them with 150 μmol/L tangeretin to further test whether this combination can enhance the growth inhibition of cisplatin-resistant cells. As shown in Fig. 1C, pretreatment of cells with tangeretin for 24 hours synergized the cytotoxic effect of cisplatin. The CI ranged from approximately 0.5 to 0.7 for every tangeretin–cisplatin combination tested. On the other hand, when cisplatin was administered before tangeretin, CI was between 1 and 1.1, indicating additive rather than synergistic effects. We selected two cisplatin doses (3 and 6 μmol/L) that kill about 20% to 30% of resistant cells and combined them with 150 μmol/L tangeretin to further test whether this combination can enhance the growth inhibition of cisplatin-resistant cells. Figure 2A shows that pretreatment of cells with tangeretin increased the potency of cisplatin over 2-fold. For instance, single treatment of cells with 3 and 6 μmol/L cisplatin caused approximately 19% and 31.4% growth inhibition in A2780, respectively. However, the growth inhibition increased to 60% and 72.9% when the cells were pretreated with tangeretin. For the drug administration sequences, using cisplatin
before tangeretin, the growth inhibition was only 42.1% and 57.9% for 3 and 6 μmol/L cisplatin, respectively. A similar response was seen with 2008/C13 cells. Treatment of 2008/C13 cells with 3 and 6 μmol/L cisplatin caused about 21% and 33% inhibition of cell survival. The inhibition was increased further from 41% and 49% to 58% and 66%, respectively, upon tangeretin pretreatment compared with when tangeretin was added 24 hours after cisplatin administration. These effects were confirmed by colony forming assay (Supplementary Fig. S2). Taken together, the results indicated that the combination of tangeretin with low dose of cisplatin elicits significantly higher cytotoxic response in cisplatin-resistant human ovarian cancer cells.

**Tangeretin enhances cisplatin-induced apoptosis via a caspase-dependent mechanism.** Cellular apoptosis was assessed first by gel electrophoretic analysis of internucleosomal DNA fragmentation as well as by a quantitative ELISA assay measuring cytoplasmic histone/DNA fragments. Treatment of A2780/CP70 and 2008/C13 cells with single exposures of tangeretin or cisplatin showed the presence of mostly high molecular weight DNA as seen with untreated control. However, a DNA ladder pattern, the typical feature of apoptosis, was distinctly observed upon combination of tangeretin with different concentrations of cisplatin. This apoptotic response was reversed upon pretreatment with the general caspase inhibitor, z-VAD-FMK (Fig. 2A, lanes 5, 6, 13, and 14 versus lanes 7, 8, 15, and 16). The quantitative measurement of apoptosis showed that tangeretin alone caused negligible increase in cell death over the background. Also, treatment with cisplatin up to 6 μmol/L revealed a minimal extent of cell death. On the other hand, tangeretin before cisplatin treatment caused a robust increase in apoptosis index of treated cells (Fig. 2B, columns 5, 6, 13, and 14) that was reversed upon caspase inhibition (Fig. 2B, columns 7, 8, 15, and 16). Moreover, the cleavage of PARP, a hallmark of caspase-dependent apoptosis, and activation of the caspase-7 and caspase-3 were also seen in parallel for the same combination treatments. Consistent with cell growth inhibition, the cleavage of caspase-7, caspase-3, and PARP were abolished by z-VAD-FMK, indicating that the apoptosis was specifically mediated through activation of the caspase pathway. Notably, the overall apoptotic response was significantly higher in combined tangeretin and 3 μmol/L cisplatin dually exposed than in 6 μmol/L cisplatin singly treated cells. Thus, these results also suggested a synergized apoptotic effect of tangeretin-cisplatin combination on cisplatin-resistant A2780/CP70 and 2008/C13 cells.

**Tangeretin-cisplatin combination arrests resistant cells in G2-M phase.** Asynchronously growing A2780/CP70 cells, treated with 150 μmol/L tangeretin followed by 3 μmol/L cisplatin, were examined for their cycle progression by flow cytometry (Fig. 3A and B). In untreated control, the percentage of cells in G1, S, and G2–M phases were 63.47%, 21.22%, and 10.51%, respectively. Single exposure with cisplatin had no significant effect on the cell cycle, whereas tangeretin caused a small redistribution to G2–M-phase. The tangeretin-cisplatin treatment, however, resulted in a pronounced G2–M arrest. The percentage of G1, S, and G2–M-phase cells following tangeretin-cisplatin treatment was 15.95%, 14.82%, and 51.98%, respectively. Thus, the accumulation of G2–M-phase cells was largely at the expense of cells failing to cycle into G1.
The total Akt level, however, remained unaffected by all treatment comparisons with that of untreated as well as singly treated controls. A2780/CP70 and 2008/C13 cells treated with tangeretin-cisplatin, (Fig. 4) showed a specific reduction in the p-Akt protein in pathway by tangeretin-cisplatin treatment. Western blot analysis way (4), we wanted to determine the potential attenuation of this ovarian cancer is related to activation of PI3K/Akt signaling path-

Because cisplatin resistance of human cisplatin combination.

Next, we investigated the functional relevance of Akt to sensiti-

A2780/CP70 cells were transfected with Akt siRNA before drug treatment and quantitative Akt knockdown was confirmed by Western blot analysis. Akt knockdown induced a dose-dependent conditions. Relatively insignificant decrease of p-Akt by tangeretin was consistent with the high IC50 observed for this treatment. The expression levels of Akt downstream substrates NF-κB, BAD, and GSK-3β were also assessed. The level of NF-κB p65 showed the expected decrease by tangeretin-cisplatin combination. The combination treatment also decreased p-BAD and p-GSK-3β without affecting their nonphosphorylated forms. Taken together, these data suggested that regulation of Akt pathway is intimately associat-

**Tangeretin-cisplatin combination induces apoptosis in resistant cells by downregulating Akt signaling.** To understand whether modulation of PI3K/Akt signaling pathway is involved in tangeretin-cisplatin–induced apoptosis in resistant cells, we performed a series of experiments based on PI3K inhibition, siRNA-mediated Akt silencing, and ectopic Akt overexpression in A2780/CP70 cells. We pretreated the cells with PI3K inhibitor LY294002 before drug administration. The results showed that PI3K inhibition induced a dose-dependent PARP cleavage and DNA fragmentation in tangeretin-treated cells (Fig. 5, lanes and columns 7 and 8), indicating that PI3K acts as an upstream factor in tangeretin-induced cellular proapoptotic signaling. Moreover, pretreatment of the cells with PI3K inhibitor alongside tangeretin-cisplatin combination treatment caused a dramatic increase of PARP cleavage and DNA fragmentation (Fig. 5, lanes and columns 10 versus lanes and columns 2, 5, 7, and 9). These data clearly identified PI3K as the upstream regulatory factor for invoking the tangeretin’s effect in augmenting the cisplatin-induced cytotoxicity and apoptosis.

As expected, flow cytometry revealed the presence of a high proportion of sub-G1-phase population (apoptotic cell) in cultures treated with tangeretin-cisplatin combination. Consistent with the flow cytometric analysis, tangeretin-cisplatin treatment reduced the levels of Cdc25C and cyclin B1, whereas the p53 level was constitutively elevated in these cells (Fig. 3C).

**p-Akt and its substrates are downregulated by tangeretin-

Because cisplatin resistance of human ovarian cancer is related to activation of PI3K/Akt signaling pathway (4), we wanted to determine the potential attenuation of this ovarian cancer is related to activation of PI3K/Akt signaling path-

**Flow cytometric analysis.** Tangeretin-cisplatin combination induced apoptosis in A2780/CP70 cells by modulating Akt signaling pathway. A, PI3K inhibitor LY294002 enhances tangeretin-cisplatin combination–induced apoptosis. One hour before drug treatment, 50 μmol/L LY294002 was applied to A2780/CP70 cells. The cells were then treated with either 100 or 150 μmol/L tangeretin or vehicle for 24 h and followed by cisplatin or vehicle treatments for another 48 h. B, silencing of Akt by siRNA potentiates tangeretin-cisplatin combination to induce apoptosis in A2780/CP70 cells. The cells were transfected with 50 μmol/L Akt siRNA for 24 h and then treated with tangeretin-cisplatin combination. C, overexpression of constitutively activated Akt hinders the ability of tangeretin-cisplatin combination to induce apoptosis. Cells were transfected with Akt cDNA for 24 h, before treatment of tangeretin-cisplatin combination. For each of the above treatments, cellular PARP was determined by Western blotting (top) and apoptosis was quantitatively assessed using Cell Death ELISA (bottom). * and **, significant difference at P < 0.01 and P < 0.001 between the groups with and without LY294002 or Akt siRNA, or Akt cDNA treatment by Tukey-Kramer Multiple Comparisons Test; n = 3.
PARP cleavage and DNA fragmentation in tangeretin-treated cells (Fig. 5B, lanes and columns 7 and 8), indicating that Akt is also an upstream factor of tangeretin-induced cellular proapoptotic signaling. Akt silencing resulted in partial PARP cleavage by 3 μmol/L cisplatin treatment, which was prominently elevated upon tangeretin-cisplatin combination treatment (Fig. 5B, lanes and columns 9 and 10 versus lanes and columns 4 and 5). The results of PARP cleavage were corroborated by parallel measurements of apoptosis. These results firmly emphasize the central role of Akt in regulation of apoptotic response in resistant A2780/CP70 cells to tangeretin-cisplatin treatment.

Finally, we transfected Akt expression construct into A2780/CP70 cells to test if ectopically overexpressed, constitutively active form of Akt can block tangeretin-cisplatin–induced apoptosis. Western blotting confirmed that Akt was significantly overexpressed in A2780/CP70 cells compared with vector transfected controls (Fig. 5C). Here, the combination treatment of the controls exhibited the signature PARP cleavage and apoptosis responses. However, in cells overexpressing Akt, both PARP cleavage and DNA fragmentation were dramatically blocked in tangeretin-cisplatin–treated cells (Fig. 5C, lanes 5 and 6). These results showed that Akt expression overrode the effect of tangeretin treatment and prevented the drug-resistant cells from undergoing apoptosis. The results further suggested that the p-Akt and its downstream substrates were vital signaling pathway components responsible for tangeretin-cisplatin treatment.

GSK-3β is PI3K/Akt downstream effector of tangeretin-cisplatin–induced apoptosis. Because PI3K/Akt influences apoptosis through multiple auxiliary pathways, we wanted to assess the extent of contribution of GSK-3β in tangeretin-cisplatin–induced apoptosis. A2780/CP70 cells were pretreated with a specific GSK-3β inhibitor, SB216763, before the tangeretin-cisplatin treatment for measuring the effects on apoptosis parameters. The inhibition of GSK-3β resulted in complete blockage of tangeretin-cisplatin–induced PARP cleavage and apoptosis (Fig. 6A and B, lanes 5 and 6 versus lanes 2 and 3). Interestingly, quantitative measurement of DNA fragmentation showed that GSK-3β inhibitor blocked ~60% of apoptosis resulting from the combination treatment. These data indicated that GSK-3β is another major target of acquired cisplatin resistance through PI3K/Akt signaling pathway and its downregulation, through inactivating PI3K/Akt signaling, contributes significantly to tangeretin-cisplatin–induced apoptosis.

Discussion

Although cisplatin has been widely used for decades to treat a wide range of cancers, de novo and acquired resistance, restricting the successful use of this potent chemotherapeutic agent continues to pose a major clinical challenge. The dose escalation necessary to overcome even a small increase in cellular resistance can cause severe cytotoxicity to dose-limiting normal tissue. Thus, strategies using dual agents that act through distinct molecular mechanisms, rather than using single agents, represent the most useful alternatives for achieving higher curability with least toxicity during cancer chemotherapy. Recently, there has been an increasing interest in evaluating synergistic cancer cell cytotoxicity from combining chemotherapeutic agents with highly promising and relatively innocuous dietary flavonoids (34, 35). In this study, we investigated the potential of a dietary flavonoid, tangeretin, to sensitize the cisplatin-resistant ovarian cancer cells to cisplatin-induced cytotoxicity. We have showed that tangeretin reduces the recalcitrance of human ovarian cancer cells to low-dose cisplatin-induced cell death through downregulation of PI3K/Akt signaling pathway.

A recent report has identified tangeretin as belonging to a superior cancer-preventive flavonoid subclass that exhibits antiproliferative activity against several human cancer cell lines (20). Its antiproliferative effects have been attributed to alteration of activities related to apoptosis and cell cycle arrest. However, the underlying molecular events mediating growth inhibition, especially the basis of synergized antiproliferative effects on cancer cells, are not completely understood. Tangeretin has been reported to induce G1 cell cycle arrest in human breast and colon cancer cells (36). In human colorectal carcinoma cells, tangeretin increases the cellular expression of p21 and p27 (37). We, however, observed an accumulation of G2-M cell population in tangeretin--singly treated as well as the tangeretin-cisplatin--treated A2780/CP70 cisplatin-resistant human ovarian cancer cells (Fig. 3). Similar to our data, tangeretin has been reported to induce G2-M arrest in p53-null HL-60 promyelocytic leukemia cells (38). As G2-M arrest is typically linked to DNA damage response, we posit that pretreatment with tangeretin sensitizes these cells to respond to DNA damage especially in the context of their p53 function. The A2780/CP70 cell line initially developed their resistance to cisplatin from repeated exposures of the parental A2780 cells to challenge doses of cisplatin. Despite severe and cumulative genotoxic exposures, A2780/CP70 cells retain their wild-type p53 gene sequence. Nevertheless, these exposures
render p53 protein transcriptionally inactive in A2780/CP70 cells. For instance, DNA damage fails to induce the accumulation of p21 protein in cisplatin-resistant parental cells, and this induction is normal in cisplatin-sensitive parental cells (39).\(^1\) More importantly, A2780/CP70 cisplatin-resistant cells constitutively express p53 protein in both wild-type as well as mutant conformation as detected by conformation-specific pAb240 antibody (39). The A2780 cells, on the other hand, express functional p53 protein recognizable by pAb1620 antibody. Moreover, transfection of V143A mutant p53 into A2780 cells increase resistance of the cells to DNA-damaging agents but not to paclitaxel (40). It is possible that the inherently altered conformation of p53 associated with the abrogated p53 function occurred in A2780/CP70 cells, contributing to their cisplatin resistance (39). The changes in p53 functional status in A2780/CP70 cisplatin-resistant cells could also make them amenable to the observed G2-M arrest in response to tangeretin-cisplatin treatment.

Increased apoptosis induced by cisplatin is another feature of cellular response to combined tangeretin and cisplatin treatment, which manifests as the synergistic growth inhibitory effect on cisplatin-resistant cells. Here, we have shown that specific PI3K inhibition by LY294002 induced a tangeretin dose–dependent PARP cleavage and DNA fragmentation in the tangeretin-treated cells. Furthermore, pretreatment of the cells with PI3K inhibitor caused a dramatic increase of PARP cleavage and DNA fragmentation in tangeretin-cisplatin–treated A2780/CP70 cells. In parallel, knockdown of Akt had a similar effect on tangeretin-cisplatin--induced apoptosis. In contrast, the overexpression of constitutively active Akt expression overrids the proapoptotic effect of tangeretin-cisplatin treatment and prevented drug-treated cells from apoptosis. These results showed that PI3K/Akt signaling pathway mechanistically regulates the tangeretin-cisplatin--induced apoptosis. Besides, clear downregulation of p-Akt and its downstream substrates NF-κB, p-GSK-3β, and p-BAD were only observed in tangeretin-cisplatin--treated cells. Tangeretin treatment alone did not appreciably alter the cellular levels of these cell survival regulators. Given that tangeretin potentiates growth inhibition of cells caused by cisplatin in a drug administration sequence–dependent manner, we reason that tangeretin pretreatment could condition the cells to respond to cisplatin-induced DNA damage by muffling the constitutive activation of p-Akt. The A2780/CP70 and 2008/C13 cells express high levels of p-Akt (Fig. 4), indicating that the cells have a constitutively activated cell survival signaling. Such a scenario of activated signaling in cells would inevitably institute an enhanced survival and impose a barrier for desired killing effects of DNA-damaging therapeutic agents. Consistent with this notion, the A2780/CP70 cells are not only resistant to cisplatin, but also cross-resistant to irradiation, melphanal, and Adriamycin (41). Therefore, implementing tangeretin pretreatment would condition A2780/CP70 and 2008/C13 cells, via downregulated Akt-mediated cell survival signaling, to become increasingly susceptible to cisplatin as well as other therapeutic agents. It should be noted that tangeretin suppresses IL-1β–induced Akt activation in human lung carcinoma cells (42). Thus, it is envisaged that the downregulation of p-Akt and its downstream substrates, NF-κB, p-GSK-3β, and p-BAD, by combined treatment reflects the synergy between tangeretin and cisplatin in modulation of Akt phosphorylation (activation).

Among many downstream factors of PI3K/Akt signaling pathway, we have only examined the contribution of GSK-3β inhibition to tangeretin-cisplatin--induced apoptosis. However, GSK-3β is not the only PI3K/Akt downstream culprit factor involved in regulating cisplatin-induced apoptosis. In fact, GSK-3β inhibitor blocked only \(~60\)% of tangeretin-cisplatin--induced apoptosis. Other downstream factors of PI3K/Akt pathway could also participate and account for the rest of the observed apoptotic effect. We also observed downregulation of NF-κB, p-GSK-3β, and p-BAD by tangeretin-cisplatin treatment. At present, it is difficult to attribute the extent of individual contribution of these regulatory factors to tangeretin-cisplatin--induced apoptosis. However, it is certain that modulation of Akt activation by the combined treatment represents a major intracellular switch to mechanistically control the tangeretin-cisplatin--induced tumor cells apoptosis.

In summary, our data experimentally showed that tangeretin pre-exposure can overcome resistance of human ovarian cancer cells to growth inhibition by cisplatin. From a mechanistic standpoint, tangeretin-cisplatin combination downregulates PI3K/Akt pathway, leading to the sensitization of cancer cells to cisplatin--induced cell death through apoptosis. The data provide a firm molecular basis for the pharmacologic effect underlying the use of tangeretin as a valuable therapeutic adjuvant. Given the broad-spectrum organ safety of tangeretin, already shown in laboratory animals in vitro (43, 44), the present work should expedite the exploration and use of tangeretin in enhancing the efficacy of cisplatin in experimental animal studies as well as clinical trials, with the caveat of successfully attaining effective tangeretin doses through human consumption of citrus fruits. It would also be appealing to determine whether tangeretin interacts with additional physical and chemical therapeutic agents in killing a variety of other drug-resistant cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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1. Our unpublished data.

**References**


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