Sensitizing HER2-overexpressing cancer cells to luteolin-induced apoptosis through suppressing $p21^{WAF1/CIP1}$ expression with rapamycin

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Abstract

HER2 overexpression, which confers resistance to various therapeutic regimens, correlates with a poor clinical prognosis. In this study, we showed that luteolin, a naturally occurring flavonoid, is a potent stimulator of HER2 degradation. Luteolin effectively inhibited cell proliferation and induced apoptosis in HER2-overexpressing cancer cells. Furthermore, we found that low doses of luteolin up-regulated p21 expression and high doses of luteolin down-regulated its expression. Examination of the Akt/mammalian target of rapamycin (mTOR) signaling revealed that this signaling was only transiently inhibited by low doses of luteolin, which suggested that the inability to cause sustained Akt/mTOR inhibition may contribute to p21 induction and provide a survival advantage to HER2-overexpressing cancer cells. To test this hypothesis, we showed that the combined use of luteolin and mTOR inhibitor rapamycin prevented low doses of luteolin from inducing p21 expression, and HER2-overexpressing cancer cells would be sensitized toward luteolin-induced apoptosis. In addition, p21 small interfering RNA also increased the luteolin-induced cell death. In nude mice with xenografted SKOV3.ip1-induced tumors, luteolin significantly inhibited HER2 expression and tumor growth in a dose-dependent manner, and rapamycin further enhanced the effect of luteolin with a concomitant p21 inhibition. These results reveal an intriguing finding that suppressing p21 expression might have therapeutic implications and further suggest that combination of mTOR inhibitors may be a promising strategy to help increase the efficacy of preventive or therapeutic compounds against HER2-overexpressing tumors. [Mol Cancer Ther 2007;6(7):2127–38]

Introduction

Breast carcinoma and ovarian carcinoma are the most frequently diagnosed malignancies, and they account for one third of all cancers in women. Amplification of the *HER2* gene (alternatively known as *neu* or *erbB2*) or overexpression of HER2 protein was found in up to 30% of breast and ovarian carcinomas (1). Breast and ovarian cancer patients whose tumor cells overexpress HER2 have a poor clinical outcome, such as shorter survival or earlier relapse (2). HER2 overexpression has been shown to enhance proliferative, prosurvival, and metastatic signals in breast and ovarian cancer cell lines. HER2-mediated signaling has also been reported to result in resistance to apoptosis induced by many stimuli (3). Additionally, repressing HER2 overexpression attenuates its antiapoptotic signaling and suppresses HER2-mediated malignant phenotype. Taken together, the data from the above-mentioned studies indicate that HER2 is not only a potent oncogene but also an excellent therapeutic target in breast and ovarian cancer.

The importance of HER2 in breast and ovarian cancer led to the development of agents that aimed at reducing HER2 level or activity (4, 5). One successful example is the use of trastuzumab (Herceptin), a recombinant humanized monoclonal antibody directed against the extracellular domain of HER2, for the treatment of metastatic breast cancer. Although trastuzumab is a successful example of a rationally designed therapeutic antibody, only 30% of patients with HER2-overexpressing breast cancer respond to trastuzumab as a single agent, and the majority of patients initially responding positively to this drug subsequently develop resistance within a year (6, 7). The viral protein such as adenovirus E1A has been reported to suppress tumor growth through repressing HER2 expression (8). However, a serious concern exists for using HER2-targeting gene therapy with E1A, as E1A is a potent viral oncoprotein that can transform primary cells. Considering that HER2 is a heat shock protein 90 (Hsp90) client protein and requires interaction with Hsp90 and its chaperone to acquire proper protein function (9), the Hsp90 inhibitor such as geldanamycin provides an alternative approach to target HER2 through dissociation of HER2 from the chaperone, leading to HER2 degradation by a proteasome-dependent manner (10). Currently, the less toxic analogue of geldanamycin, 17-allyamino-17-demethoxygeldanamycin, is being actively evaluated in multiple phase II clinical trials.

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Recently, the use of phytochemicals as possible chemopreventives or chemotherapeutic agents has gained in importance (11). Way et al. (12) have shown that apigenin induces apoptosis by depleting HER2 protein in HER2-overexpressing breast cancer cells via proteasomal degradation. In an attempt to search for compounds more effective than apigenin, they examined the relationship between the chemical structure and the inhibitory effect of flavonoids of different chemical classes on the expression of HER2 protein. Among the 19 flavonoids investigated, luteolin showed the most effective inhibition of HER2 expression (13). Luteolin, a member of the flavone family, widely exists in many edible plants. Luteolin has been reported to be antitumor and antiangiogenic by selectively blocking signal transduction pathways in a variety of cancer cells. Several molecular mechanisms have been suggested for the anticancer effect of luteolin, including inactivation of multiple protein kinases (14–16). Although it has been suggested that luteolin possesses strong antineoplastic characteristics, its effect on HER2-overexpressing cancer cells has rarely been mentioned.

p21$^{WAF1/CIP1}$ (hereafter referred to as p21) was originally described as a wild-type p53-inducible gene as well as a universal inhibitor of cyclin-dependent kinases. In addition to regulating the cell cycle, many reports have suggested that p21 also plays an important role in the regulation of apoptosis (17). In various cancer cell lines, cells lacking p21 are more sensitive to apoptosis induced by a variety of methods (18–20). The exact mechanisms by which p21 can protect cells from undergoing apoptosis are not entirely clear. One of the mechanisms is assumed to involve p21-mediated cell cycle arrest (17). By arresting cell cycle progression, p21 prevents the onset of the apoptotic program. In addition, p21 could interact with and inhibit proapoptotic molecules, such as procaspase-3, caspase-8, and apoptosis signal-regulating kinase 1 (21, 22). Counterintuitive to the role of p21 in antiapoptosis, however, p21 has also been shown to function as an apoptosis-promoting protein, and the mechanisms by which p21 promote apoptosis may be related to its interaction with DNA repair machinery (23). Collectively, the role of p21 in apoptosis remains controversial and merits further investigations.

In this report, we showed that the down-regulation of HER2 expression by the flavonoid luteolin is a general phenomenon in HER2-overexpressing cancer cell lines. Furthermore, we showed that the growth inhibition effect of luteolin on HER2-overexpressing cancer cells is through the induction of apoptosis. Interestingly, we found that low doses of luteolin would induce the expression of p21, whereas high doses of luteolin would inhibit p21 expression. Reducing the protein level of induced p21 protein level by mammalian target of rapamycin (mTOR) inhibitor rapamycin or p21 small interfering RNA (siRNA) would result in stronger growth inhibition and apoptosis. These results were further confirmed in *in vivo* tumor xenograft model. Overall, inhibition of p21 expression leads to increased cell death induced by luteolin in HER2-overexpressing cancer cells.

Materials and Methods

Antibodies and Reagents

Antibodies and reagents were purchased from commercial sources: PD98059 and antibodies against phosphorylated Ser^{473} Akt, Akt, phosphorylated Ser^{2248} mTOR, phosphorylated Thr^{389} S6 protein kinase 1, p21, cleavaged caspase-3, poly(ADP-ribose) polymerase (PARP), and cleavaged PARP were purchased from Cell Signaling Technology; antibodies against *erbB2* (Ab3) was from Oncogene Science; antibodies against Hsp90, cyclin D1, and p27 were from BD Biosciences; protein A/G-agarose and anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology; and β-actin antibody, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wortmannin, LY294002, MG132, luteolin, and rapamycin were from Sigma Chemical Co.

Cell Lines and Cell Cultures

The human breast and ovarian cancer cell lines used in this study were MDA-MB-453, AU565, SKOV3.ip1, HBL-100, and MCF-7. MCF-7 and AU565 were cultured in DMEM supplemented with 10% FCS (Hyclone Laboratories) and 1% penicillin-streptomycin, and other cell lines were cultured in DMEM/F-12. These cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of Cell Lysate, Immunoblotting, and Immunoprecipitation

Cells were treated with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer [1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 25 mmol/L NaF, 0.5 mmol/L sodium orthovanadate, 1 mmol/L DTT, 1 μg/mL pepstatin, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.1 mg/mL phenylmethylsulfonyl fluoride]. For immunoprecipitation, 1 mg of each sample was mixed with 2 μg antibody and 25 μL protein A/G-agarose at 4°C for 3 h. The immunoprecipitates were washed with lysis buffer and eluted with the SDS sample loading buffer and processed for immunoblotting analyses as described previously (12, 13).

For preparation of Triton X-100–soluble and Triton X-100–insoluble fractions, cells were lysed with lysis buffer containing 1% Triton X-100 as described above. After removal of Triton X-100–soluble cell lysate supernatants by centrifugation, the pellets were washed once with the lysis buffer and 1× SDS loading buffer (50 μL) was then added to the pellets and heated at 95°C for 15 min to dissolve the Triton X-100–insoluble proteins.

Reverse Transcription-PCR

Total RNA was isolated by the Isogen reagent (Nippon Gene). cDNA was prepared from 5 μg of total RNA with Moloney murine leukemia virus reverse transcriptase and oligo(dT)$_{18}$ primer. The PCR was done in a final volume of 50 μL, which contained 4 μL of deoxynucleotide triphosphates, 1 μL of reaction buffer, 1 μmol/L of each primer (HER2: forward, 5′-CTGCAACACCTTCTGCAGTTCTG-3′;

reverse, 5′-TCGAATTTGCCAATTTCCAGGAAGC-3′), 2 μL of cDNA, and 50 units/mL of Taq DNA polymerase. Each 5 μL PCR product was separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Cell Proliferation Assays and Flow Cytometry

As described previously (12), the effects of luteolin and rapamycin on cell proliferation were examined by MTT method, and the cell cycle analysis of the sub-G_1 peak detection of the apoptotic effect was determined by flow cytometry using propidium iodide staining.

Transient Transfections

p21 siRNA#1 was designed to target specific sequences of human p21 (accession number NM000389; sequence, 5′-ACAAAGUCGAAGUUCCAUCUU-3′). p21 siRNA#2 and control siRNA were purchased from Cell Signaling Technology. Plasmid pCMV-p21 was a kind gift from Prof. Zee-Fen Chang (National Taiwan University, Taipei, Taiwan). One day before transfection, cells were seeded in six-well plate without antibiotics with the density of 30% to 40%. For siRNA transfections, p21 and control siRNAs were premixed with LipofectAMINE 2000 in Opti-MEM and then added to each well for 24 h. For plasmid transfections, 2 μg of plasmid DNA were premixed with LipofectAMINE 2000 in Opti-MEM and added to wells for 6 h.

In vivo Studies

Female BALB/c nude mice (18–20 g; 6–8 weeks of age) were purchased from the National Animal Center and maintained in pressurized ventilated cage according to institutional regulations. SKOV3.ip1 cells (2×10^6) were inoculated s.c. into the right flank of the mice. After 7 days, 25 tumor-bearing mice were randomly divided into five groups for treatment with luteolin and/or rapamycin. The first group only received vehicle. The second to fifth groups were given i.p. the following treatments every 3 days,

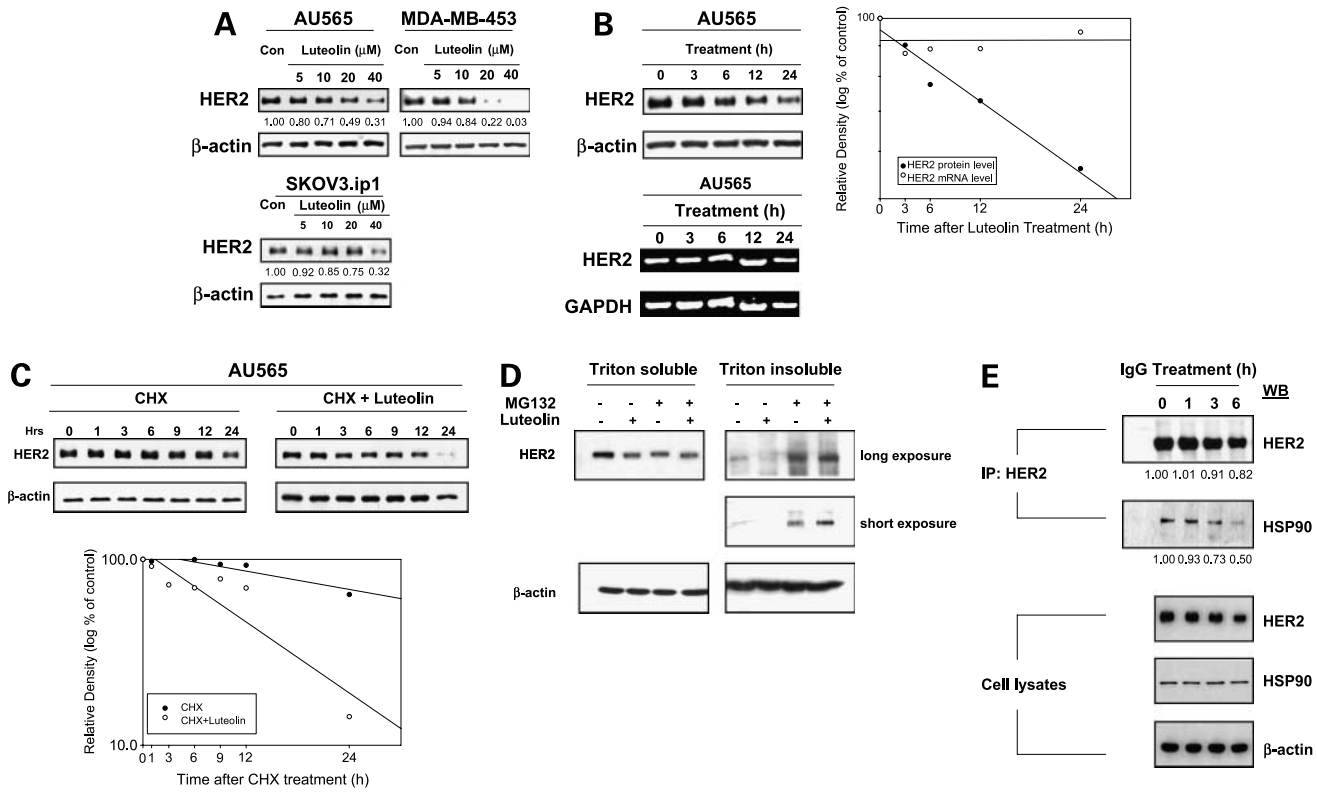

Figure 1. Influence of luteolin on the level of HER2 in HER2-overexpressing cancer cell lines. **A,** HER2-overexpressing breast cancer cell lines (AU565 and MDA-MB-453) and ovarian cancer cell line (SKOV3.ip1) were treated with luteolin (5, 10, 20, and 40 μmol/L) at 37°C for 24 h. Immunoblotting was used to measure HER2 and β-actin. *Values below the figures,* change in the protein expression of the bands normalized to β-actin. **B,** AU565 cells were incubated with DMSO or luteolin (40 μmol/L) at 37°C for various times. *Top,* protein level of HER2 and β-actin in AU565 cells was analyzed by Western blotting; *bottom,* mRNA level of HER2 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in AU565 cells was analyzed by reverse transcription-PCR. *Right,* relative changes in HER2 protein and mRNA levels from the averaged results of three independent experiments. **C,** luteolin decreased the half-life of HER2. AU565 cells were cultured with 20 μg/mL cycloheximide (*CHX*) in the presence or absence of 40 μmol/L luteolin for the indicated times. *Top,* representative experiment in which β-actin and HER2 protein levels were assessed by Western blot analysis; *bottom,* quantification of HER2 expression normalized to the level of β-actin control. HER2 expression at the 0-h time point was set as 100%. **D,** MDA-MB-453 cells were pretreated with MG132 (20 μmol/L) for 30 min followed by 40 μmol/L luteolin for 8 h, and Triton X-100–soluble and Triton X-100–insoluble cell lysates were prepared and assessed by immunoblotting with antibodies to HER2 and β-actin. **E,** dissociation of Hsp90-HER2 complex by luteolin in MDA-MB-453 cells. Cells were treated with 40 μmol/L luteolin for the duration indicated. Cell lysates were subjected to immunoprecipitation with a mouse monoclonal anti-HER2 antibody or with a mouse monoclonal antibody (IgG, used as a control for potential nonspecific binding). *Top,* immunoprecipitates were analyzed by immunoblotting with an anti-HER2 or anti-Hsp90 antibody. *Values below the figures,* quantification of the bands normalized to control. *Bottom,* an aliquot of each cell lysate used for the immunoprecipitation was examined by immunoblotting with antibodies to HER2, Hsp90, and β-actin.

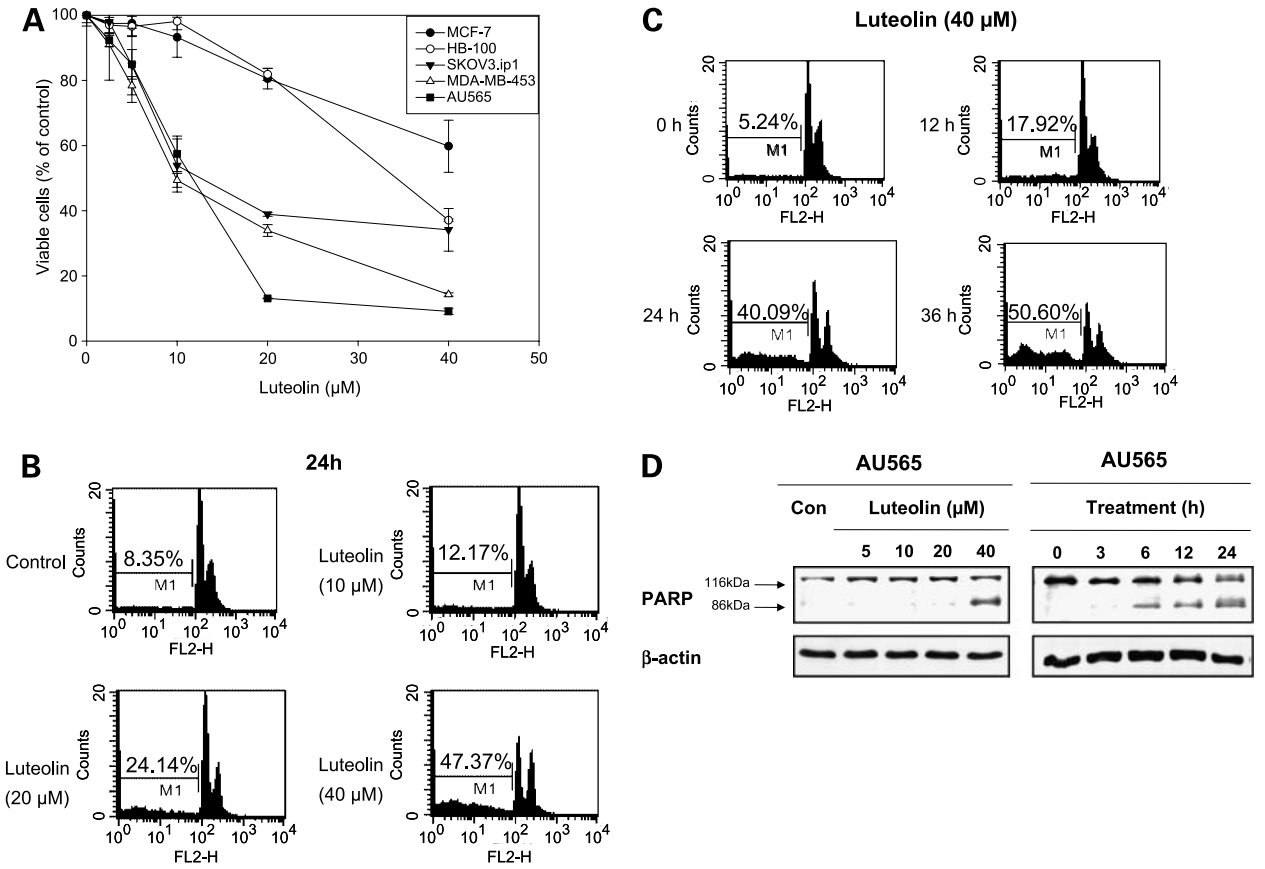

Figure 2. Effects of luteolin on the inhibition of proliferation and induction of apoptosis in HER2-overexpressing cancer cells. **A,** cell viability was determined by MTT assays after continuous exposure to different concentrations of luteolin at 37°C for 72 h. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. *Points,* mean of three independent experiments; *bars,* SD. **B** and **C,** sub-G_1 peak detection of the apoptotic effect was analyzed by flow cytometry. AU565 cells were treated with the indicated concentrations of luteolin for 24 h (**B**) or 40 μmol/L luteolin for 12, 24, and 36 h (**C**). Data are representative of two independent experiments. Percentage of cells with sub-G_1 DNA content in each histogram. **D,** AU565 cells were treated with the indicated concentrations of luteolin for 24 h (*left*) or 40 μmol/L luteolin for 3, 6, 12, and 24 h (*right*). PARP cleavage was assessed by Western blot analysis. *Top band,* uncleaved PARP; *bottom band,* cleaved PARP.

respectively: luteolin (5 mg/kg), luteolin (50 mg/kg), rapamycin (1 mg/kg), and luteolin (5 mg/kg) + rapamycin (1 mg/kg). Mice were weighed and tumors were measured using calipers every 3 days. Tumor size was calculated with the following formula: $(L + W) / 2$, where L is the length and W is the width. On the final day of the treatment, mice were sacrificed; tumors were excised, weighted, and sectioned; and the tumor sections were embedded in OCT compound and frozen at −70°C.

Immunohistochemical Staining of Frozen Tissue Sections

Sections frozen in OCT were fixed in acetone and chloroform. After overnight incubation with primary antibodies, including (*a*) mouse monoclonal anti-HER2/*neu* (Ab3, 1:300 dilution; Oncogene Science) and (*b*) rabbit polyclonal anti-p21 (c-19, 1:100 dilution; Santa Cruz Biotechnology), the slides were washed again and then incubated with biotinylated secondary antibodies and subsequently incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories). Antibody detection was done with 3,3′-diaminobenzidine, and the tissue sections were counterstained with Mayer's hematoxylin, washed, mounted with Universal Mount, and dried on a 56°C hot plate. The prepared slides were examined by light microscopy.

Statistical Analysis

All values were expressed as mean ± SD. Each value is the mean of at least three separate experiments in each group. Student's t test was used for statistical comparison. Asterisk indicates that the values are significantly different from the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The Bliss additivism model was used to classify the effect of combining rapamycin and luteolin as additive, synergistic, or antagonistic as described previously (24).

Results

Luteolin Promotes Degradation of HER2

It has been shown previously that the expression of HER2 protein is down-regulated by luteolin in MDA-MB-453 cell line (13). To further confirm that the inhibitory

effect of luteolin on the HER2 protein level is a general phenomenon for HER2-overexpressing cancer cell line, we examined the effect of luteolin on the HER2 protein level in AU565, MDA-MB-453, and SKOV3.ip1 cells with various concentrations of luteolin at 37°C for 24 h, and HER2 protein level was measured by Western blot. These results show that luteolin suppresses HER2 expression in a dose-dependent manner (Fig. 1A).

To better delineate the mechanism of luteolin-mediated HER2 down-regulation, we tested the effect of luteolin on HER2 protein level compared with mRNA level. The HER2 protein levels decreased in a time-dependent manner after luteolin treatment, whereas HER2 mRNA level did not significantly decline even after 24 h (Fig. 1B). Similar results were also observed in MDA-MB-453 cells (Supplementary Fig. S1).[3] These results suggest that the luteolin-reduced HER2 expression is through posttranscriptional mechanism. To determine whether HER2 degradation is accelerated by luteolin, we treated AU565 cells with translation inhibitor cycloheximide or with the inhibitor plus luteolin and then measured the relative HER2 level in these cells. As shown in Fig. 1C, the HER2 level decreased faster in cells treated with cycloheximide plus luteolin than in cells treated with cycloheximide alone. This result suggests that a posttranslational mechanism contributes to luteolin-induced HER2 depletion in HER2-overexpressing cancer cells. To further show the role of proteolysis in luteolin-mediated HER2 down-regulation, we carried out studied with the proteasome inhibitor MG132. In the absence of MG132, luteolin reduced the levels of HER2 in both detergent (Triton X-100)–soluble and detergent (Triton X-100)–insoluble cellular fractions. MG132 treatment inhibited luteolin-mediated decrease of HER2 levels in the Triton X-100–insoluble cellular fraction (Fig. 1D). These results suggest that proteasomal activity is involved in luteolin-induced HER2 degradation.

Dissociation of HER2 from Hsp90 Precedes the Depletion of HER2

HER2 is bound to the Hsp90 molecular chaperone complex, which is essential for HER2 stability and maturation (25). To further study the mechanism of HER2 depletion, we treated MDA-MB-453 cells with 40 μmol/L luteolin for short durations (1–6 h) to minimize the difference in the HER2 protein level among samples and studied the binding of HER2 and Hsp90. Through immunoprecipitation experiments, we found that luteolin disturbed the binding of Hsp90 to HER2 without significantly affecting the levels of Hsp90 (Fig. 1E). These results suggest that luteolin diminished HER2 levels by interfering with the binding of HER2 to Hsp90.

Luteolin Preferentially Inhibits the Proliferation of HER2-Overexpressing Cancer Cells

To assess the biological activity of luteolin in terms of cell proliferation, cells were treated with luteolin at different concentrations for 3 days. The growth inhibition of the tested cell lines was in a dose-dependent manner but to various extents (Fig. 2A). For example, luteolin at 20 μmol/L blocked >60% of growth in HER2-overexpressing cancer cells (AU565, MDA-MB-453, and SKOV3.ip1). However, the inhibition was much less effective for those cells expressing a basal level of HER2 (MCF-7 and HBL-100) under the same condition. Overall, these results suggest that luteolin preferentially suppresses the growth of HER2-overexpressing cancer cells.

Luteolin Promotes Apoptotic Cell Death in HER2-Overexpressing Cancer Cells

The percentage of apoptotic cells in the sub-G_1 peak of luteolin-treated AU565 cells was measured by flow cytometry. As shown in Fig. 2B and C, a significant number of the cells (~40%) started to undergo apoptosis as early as 24 h after the treatment with 40 μmol/L luteolin. PARP, cleaved by caspase-3 during apoptosis to produce M_r 86,000 fragments from the full-length M_r 116,000 protein, was measured as a marker of apoptosis by

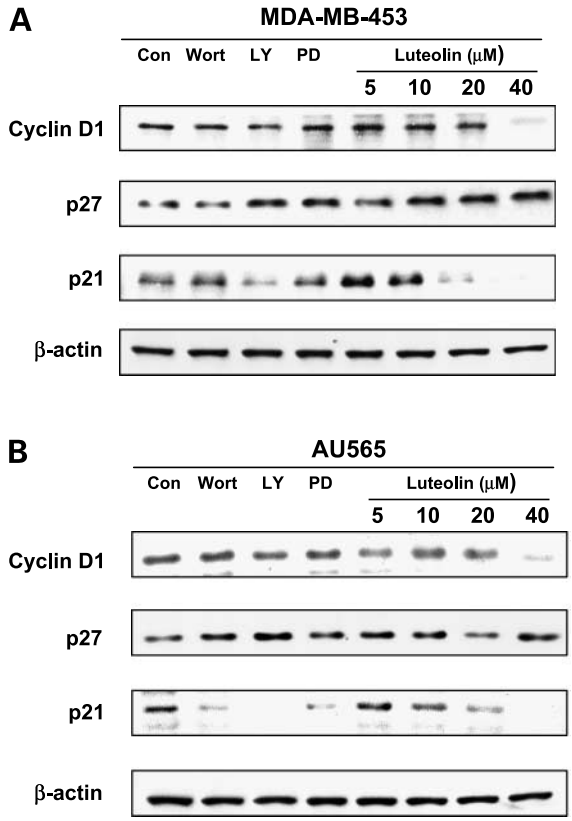

Figure 3. Effects of luteolin on the cell cycle regulatory proteins in HER2-overexpressing cancer cells. **A,** MDA-MB-453 cells were treated with the phosphatidylinositol 3-kinase inhibitors wortmannin (*Wort*; 500 nmol/L) or LY294002 (*LY*; 25 μmol/L) or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 inhibitor PD98059 (*PD*; 25 μmol/L) or luteolin (5, 10, 20, and 40 μmol/L) at 37°C for 24 h. Levels of cyclin D1, p27, p21, and β-actin were analyzed by Western blotting. **B,** AU565 cells were treated and analyzed as described in **A**.

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

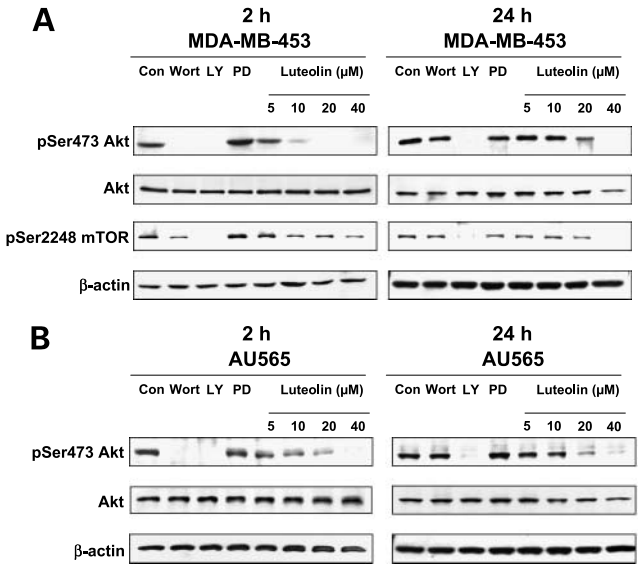

Figure 4. **A,** MDA-MB-453 cells were treated with the phosphatidylinositol 3-kinase inhibitors wortmannin (500 nmol/L) or LY294002 (25 μmol/L) or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 inhibitor PD98059 (25 μmol/L) or luteolin (5, 10, 20, and 40 μmol/L) at 37°C for 2 or 24 h. Levels of phosphorylated Ser^{473} (*pSer473*) Akt, Akt, phosphorylated Ser^{2248} (*pSer2248*) mTOR, and β-actin were analyzed by Western blotting. **B,** AU565 cells were treated and analyzed as described in **A**.

immunoblotting. Luteolin induced corresponding increases in the PARP cleavage (Fig. 2D). This indicated that luteolin-treated AU565 cells underwent apoptosis in a dose- and time-dependent manner. Similar results were obtained in other cell lines overexpressing HER2 (MDA-MB-453 and SKOV3.ip1; Supplementary Fig. S2; data not shown).[3] Therefore, induction of apoptosis could be a major mechanism of luteolin-induced growth inhibition in HER2-overexpressing cancer cells.

Luteolin Alters Cell Cycle Regulatory Proteins in HER2-Overexpressing Cancer Cells

Recent studies have shown that modulation of both cyclin D1 and p27 is required for oncogenic growth driven by HER2 (26, 27). To address whether these cell cycle regulatory proteins were affected by luteolin, protein levels were determined by immunoblotting. As shown in Fig. 3A, there was a notable decrease in the steady-state level of cyclin D1 in HER2-overexpressing MDA-MB-453 cells. In contrast, the p27 protein level had increased. It is also worth noting that the p21 level was increased by low doses of luteolin and repressed by high doses of luteolin. To further confirm that these changes are general phenomena, the other HER2-overexpressing cancer cell line, AU565, was analyzed, and similar results revealed (Fig. 3B). Interestingly, the biphasic changes in the p21 level induced by different doses of luteolin were not observed in cancer cells that express a basal level of HER2 (data not shown), suggesting that such phenomenon only occurs in HER2-overexpressing cancer cells.

Low Doses of Luteolin Fail to Induce Sustained Inhibition of Akt/mTOR Signaling in HER2-Overexpressing Cancer Cells

It has been shown that up-regulation of p21 protects cells against drug-induced cytotoxicity in HER2-overexpressing cancer cells (28, 29). We speculated that induction of p21 expression by low doses of luteolin may provide a survival advantage for HER2-overexpressing cancer cells and protect cells from luteolin-induced cell death. HER2 overexpression results in robust phosphatidylinositol 3-kinase/Akt activation, leading to dysregulated cell proliferation and enhanced cell survival (30). Thus, we would like to examine whether the different mode of effects of different doses of luteolin on the p21 expression in HER2-overexpressing cancer cells resulted from alteration of the Akt signaling pathway. As shown in Fig. 4A, although low-dose luteolin inhibits Akt and its downstream mTOR phosphorylation, this inhibition was not durable. After 24 h of low-dose luteolin treatment, Akt/mTOR signaling resumed despite a transient inhibition at 2 h. Treatment with high doses of luteolin, however, the Akt signaling failed to resume. Similar results were observed in AU565 (Fig. 4B). Wortmannin and LY294002, known to be irreversible phosphatidylinositol 3-kinase inhibitors, were used here as positive controls. Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 inhibitor PD98059 was used as a negative control. Treatment of the HER2-overexpressing breast cancer cells with wortmannin almost inhibited Akt phosphorylation at 2 h, whereas the reduced inhibition occurred at 24 h after treatment. This is presumably due to the short half-life of wortmannin.

Rapamycin Inhibits Luteolin-Induced p21 Expression

Low doses of luteolin cannot induce sustained inhibition of Akt signaling that might limit their antitumor activity, and it suggests that a combination of signal transduction inhibitor will probably have greater effect. An earlier report showed that a rapamycin (mTOR inhibitor) derivative, RAD001, sensitizes tumor cells to cisplatin-induced apoptosis by suppressing p21 translation (31). Therefore, we tested whether rapamycin could inhibit the p21 expression induced by low doses of luteolin and sensitize luteolin-induced cytotoxicity. The analysis of p21 protein levels in MDA-MB-453 and AU565 cells following luteolin and rapamycin treatment showed that the increase in p21 by low doses of luteolin was then strongly inhibited by rapamycin (Fig. 5A). Inhibition of mTOR activity in cells treated with rapamycin was confirmed by measuring the phosphorylation of S6 protein kinase Thr^{389}.

Rapamycin Enhances the Growth-Inhibitory Effects of Luteolin

The combination of rapamycin and luteolin exhibited greater dose- and time-dependent inhibition of cell proliferation than did each agent alone (Fig. 5B). For example, concurrent treatment of the MDA-MB-453 cells with luteolin (5 μmol/L) and rapamycin exerted a stronger inhibition on cell growth than luteolin alone, accounting for 11% to 21%, 17% to 44%, and 24% to 56% growth inhibition after 24, 48, and 72 h of treatment, respectively. Similar

results were also observed in AU565 and SKOV3.ip1 cells (Fig. 5C; data not shown). These data suggest that the combination of luteolin and rapamycin, compared with luteolin, is much more potent in inhibiting the growth of HER2-overexpressing cancer cells.

Rapamycin Increases Luteolin-Induced Apoptosis

Flow cytometric cell cycle analysis was done to determine whether the results from the MTT assays were a reflection of apoptosis. After 48 h of drug treatment, cells were fixed and the DNA content was measured in comparison with

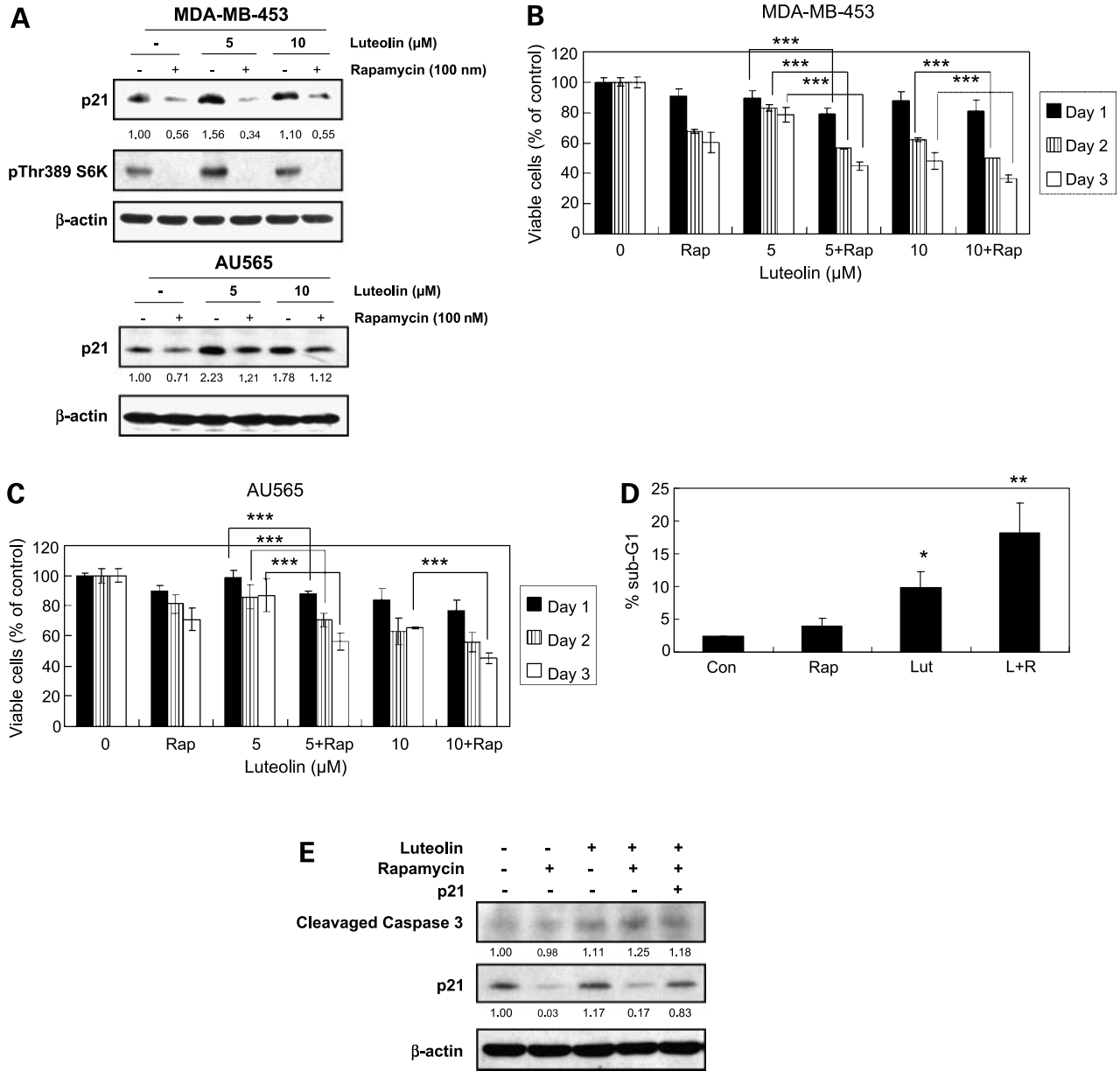

Figure 5. Rapamycin enhanced luteolin-induced growth inhibition and apoptosis in HER2-overexpressing cancer cells. **A,** rapamycin inhibits luteolin-induced p21 expression in HER2-overexpressing cancer cells. MDA-MB-453 and AU565 cells were treated for 24 h with the indicated concentrations of luteolin in the presence of DMSO or 100 nmol/L rapamycin. Levels of p21, phosphorylated Thr^{389} (*pThr389*) S6 protein kinase (*S6K*), and β-actin were assessed by Western blotting. *Values below the figures,* relative changes in p21 protein level normalized to β-actin. **B,** HER2-overexpressing MDA-MB-453 cells were treated for various times with either DMSO or 100 nmol/L rapamycin (*Rap*) in combination with the indicated concentrations of luteolin. Loss of cell viability was measured using the MTT assay. ***, $P < 0.001$, differences between groups considered statistically significant. **C,** AU565 cells were treated and analyzed as described in **B. D,** MDA-MB-453 cancer cells were grown in the presence or absence of rapamycin (100 nmol/L) in combination with 10 μmol/L luteolin (*Lut*) for 48 h. Cells were harvested for DNA content analysis. *Columns,* mean of three independent experiments; *bars,* SD. Asterisk, values significantly different from the control. *, $P < 0.05$; **, $P < 0.01$. **E,** 1×10^6 MDA-MB-453 cells were transiently transfected with 2 μg of pCMV-p21 plasmid or control pCMV plasmid. The following day, luteolin (10 μmol/L) or rapamycin (100 nmol/L) was added for an additional 48 h where indicated. The levels of p21, cleavaged caspase-3, and β-actin were assessed by immunoblotting with indicated antibodies.

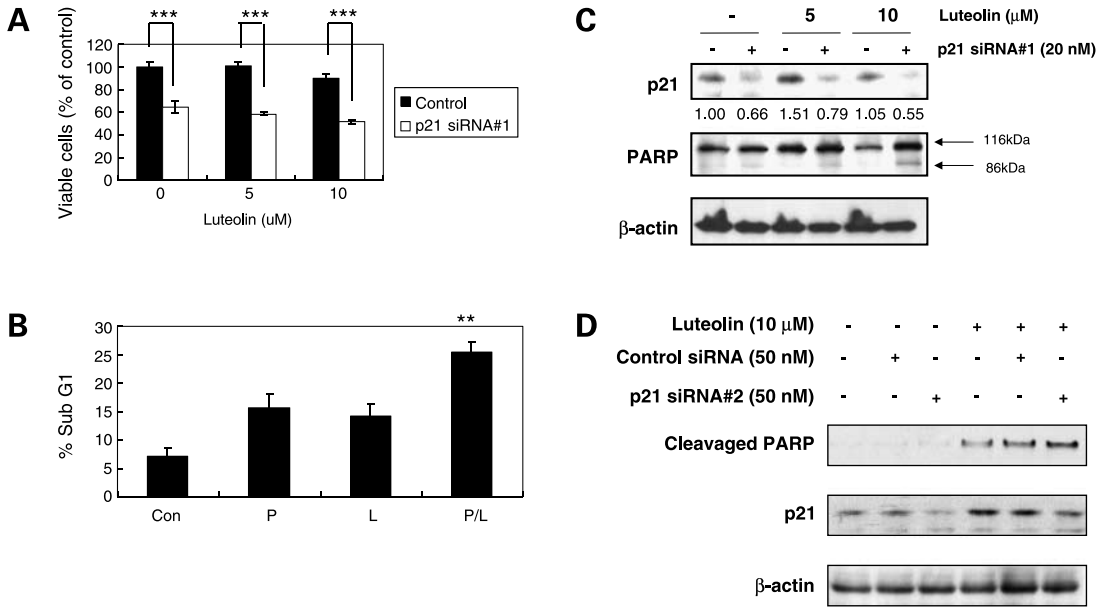

Figure 6. p21 siRNA enhanced luteolin-induced growth inhibition and apoptosis in HER2-overexpressing cancer cells. **A** to **C,** AU565 cells were untransfected or transfected with p21 siRNA#1 as described in Materials and Methods. Cells were then treated with indicated doses of luteolin for 24 h. **A,** cell viability was determined by MTT assay, and the number of viable cells after treatment is expressed as a percentage of the control. *Columns,* mean of three independent experiments; *bars,* SD. ***, $P < 0.001$, Student's *t* test. **B,** sub-G_1 peak detection of the apoptotic effect was analyzed by propidium iodide DNA staining and flow cytometry. The sub-G_1 DNA contents were plated as histogram. *Columns,* mean of three independent experiments; *bars,* SD. Asterisk, values significantly different from the control. **, $P < 0.01$. **C,** protein levels of PARP, p21, and β-actin were assessed by Western blotting. The relative p21 expression after normalization to β-actin was indicated. **D,** AU565 cells were transfected with control siRNA or p21 siRNA#2. Cells were incubated for an additional 24 h, and 10 μmol/L luteolin was added. After another 24 h, protein levels of cleavaged PARP, p21, and β-actin were assessed by Western blotting.

untreated cells (Fig. 5D). Luteolin alone induced a 4-fold increase in the sub-G_1 cell population, whereas rapamycin treatment produced only a slight increase. Together, luteolin and rapamycin induced an 8-fold increase. Consistent with this observation, the combination of luteolin and rapamycin induced corresponding increases in the caspase-3 cleavage, and ectopic overexpression of p21 reduced the cleavage (Fig. 5E). These data confirmed that treatment with a combination of luteolin and rapamycin increased apoptosis in cells. Overall, these data suggested that the enhanced cytotoxicity achieved by this drug combination is due in part to apoptosis.

p21 siRNA Promotes Luteolin-Induced Cell Growth Inhibition and Cell Death

The above-mentioned findings have suggested that rapamycin enhances the ability of luteolin to induce cell death through inhibition of p21 expression. We used a p21 siRNA approach for inhibition of luteolin-mediated p21 induction to determine the effect of p21 inhibition on luteolin-induced apoptosis. A combination of siRNA-targeting p21 and low doses of luteolin treatment strikingly promoted growth inhibition (Fig. 6A) and sub-G_1 fractions (Fig. 6B) in comparison with either treatment alone. This result was further confirmed by the fact that the increased sub-G_1 fraction correlated with the appearance of the PARP cleavage products (Fig. 6C and D), a relationship that is highly consistent with the induction of apoptosis. This observation suggests that p21 expression plays a critical role in cell viability and that the induction of p21 expression by low doses of luteolin is a potential drug resistance factor leading to cell survival.

Growth Inhibition of SKOV3.ip1 Cells *In vivo*

After enhancing the sensitivity to luteolin in HER2-overexpressing cancer cells with rapamycin *in vitro*, the effects of luteolin and rapamycin, alone and in combination, were examined *in vivo*. Twenty-five female nude mice were individually injected s.c. with SKOV3.ip1 cells. One week after inoculation, the mice were divided into five groups (five mice per group) and treated with vehicle alone, luteolin alone (5 or 50 mg/kg), rapamycin alone (1 mg/kg), or a combination of luteolin (5 mg/kg) and rapamycin (1 mg/kg). As shown in Fig. 7A, this *in vivo* tumor model showed a significant reduction in tumor volume in mice treated with 50 mg/kg luteolin when compared with control mice ($P < 0.001$). Furthermore, mice treated with 5 mg/kg luteolin-rapamycin combination showed a significant tumor size reduction when compared with mice treated with 5 mg/kg luteolin alone ($P < 0.001$) and rapamycin alone ($P = 0.02$). These results showed that luteolin significantly inhibited SKOV3.ip1 tumor growth in a mouse xenograft model and that rapamycin enhanced the efficacy of luteolin *in vivo*.

Immunohistochemical Analysis of Tumor Sections for HER2 and p21

To determine whether HER2 is targeted by luteolin *in vivo* and to determine if the results seen in cell culture

about inhibition of p21 expression would occur in tumors from animals treated with rapamycin, on the final day of the SKOV3.ip1 antitumor experiment, tumor sections were stained separately with HER2 and p21 to determine if the proteins in the tumors were altered. Representative immunohistochemical photographs of HER2 and p21 are shown in Fig. 7B. In the control group, HER2-positive cells showed a red or light brown membranous signal (Fig. 7B, *a*). Treatment with luteolin dose dependently inhibited the expression of HER2 in SKOV3.ip1 cells (Fig. 7B, *b* and *c*). The combination of luteolin and rapamycin further reduced HER2 staining when compared with using luteolin alone (Fig. 7B, *b* and *e*). Compared with control group (Fig. 7B, *f*), p21 was induced by low doses of luteolin (Fig. 7B, *g*) and inhibited by high doses of luteolin (Fig. 7B, *h*). As with the cell work, whereas low-dose luteolin showed strong staining for p21, treatment or combination with rapamycin showed virtually no induction of p21 staining compared with the control group (Fig. 7B, *f*, *i*, and *j*). These findings indicate that rapamycin can, through the down-regulation of p21 expression, enhance the sensitivity of HER2-overexpressing cancer cells to luteolin both *in vitro* and *in vivo*.

Discussion

Apoptosis is said to be one of the main mechanisms through which flavonoids inhibit the growth of cancer cells. Luteolin has been shown to cause cell death by up-regulation of DR5 (32), activation of p53 (33), inhibition of fatty acid synthase activity (34), cleavage of Bcl-2 family proteins (35), and promoting signal transducers and activators of transcription 3 degradation (36). We showed here that, although luteolin is very effective in depleting HER2 in tumor cells that either overexpress or express the basal level of HER2 (Fig. 1A; Supplementary S3),[3] luteolin preferentially inhibited the growth of HER2-overexpressing cancer cells comparing with cells expressing a basal level of HER2 (Fig. 2A). It has been suggested that the physiologically dependence on the continued expression of overexpressed oncogene for maintaining the cancer phenotype provides an Achilles heel for tumors that can be exploited in cancer therapy (37). This is consistent with a recent report that HER2-overexpressing cancer cells are dependent on HER2 for survival and thus are more sensitive to treatments that target HER2.

The concept of oncogene addiction suggests that cancer cell might be more susceptible to therapies that target the oncogene critical for the development of the specific cancer. In this respect, lung cancer patients who showed clinical responsiveness to the gefitinib (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor, harbor somatic gain-of-function mutations in the kinase domain of epidermal growth factor receptor (38). Despite the dramatic

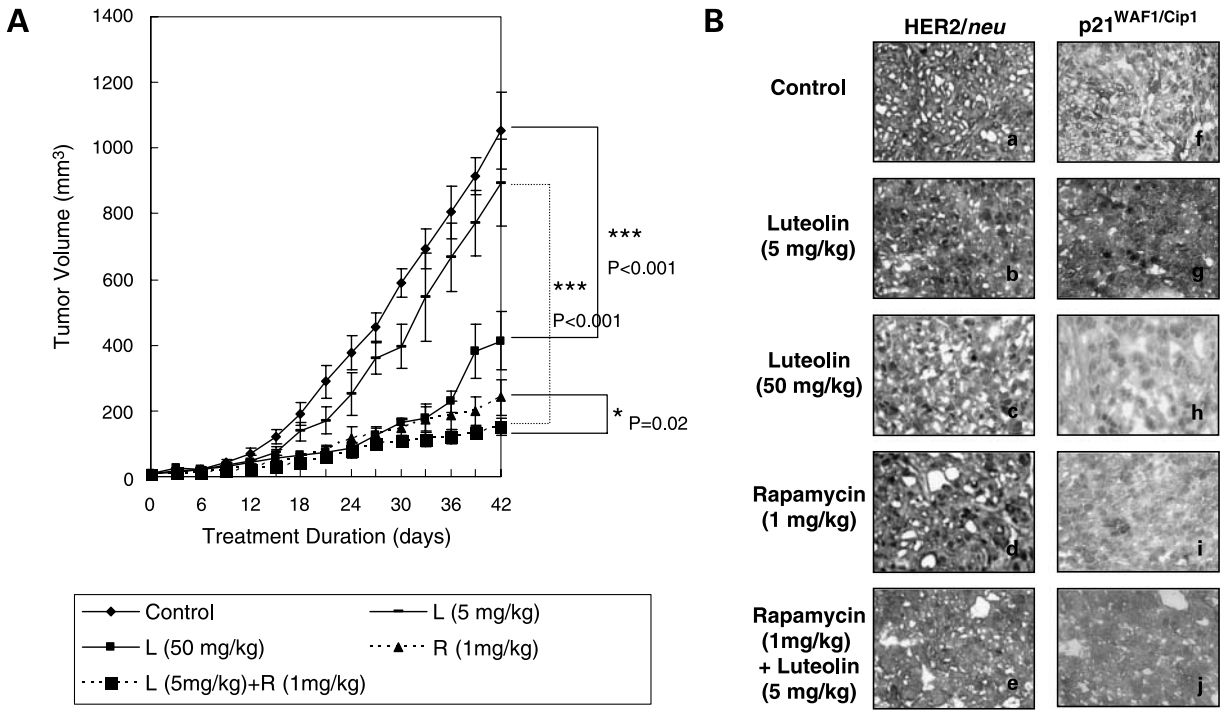

Figure 7. Effects of luteolin and rapamycin on SKOV3.ip1 tumor growth in a mouse xenograft model. **A,** female nude mice received injections of SKOV3.ip1 transfectants to induce tumor xenografts. Seven days later, mice were divided into five groups. The second to fifth groups were given i.p. with luteolin (5 mg/kg), luteolin (50 mg/kg), rapamycin (1 mg/kg), and luteolin (5 mg/kg) + rapamycin (1 mg/kg), respectively, every 3 d for 42 d. Tumor volume was measured and calculated as described in Materials and Methods. *Points,* mean tumor volume; *bars,* SD. Asterisk, values significantly different from the control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B,** removed tumors were stained with antibodies to HER2 and p21. *a* to *e,* HER2 immunohistochemical staining of tumor slides from different treatment groups (groups 1 – 5). Magnification, ×200. *f* to *j,* p21 immunohistochemical staining of tumor slides from different treatment groups (groups 1 – 5). Magnification, ×400.

response to gefitinib in lung cancer patients carrying mutant epidermal growth factor receptor, however, gefitinib resistance can arise, and most patients who initially responded to gefitinib treatment ultimately have a relapse (39). It is likely that cancer cells acquire multiple mutations during the process of tumor development, and targeting only one alteration will lead to the emergence of drug-resistant mutations or of cell variants (40). Thus, combinations of drugs will probably have a stronger inhibitory effect. Considering that the difficulties of trastuzumab in clinical use are in the time-dependent development of tumor resistance to therapy and the nonspecific toxicity toward normal heart cells (6, 7), it is suggested that inhibiting only HER2 is very unlikely to block the malignant process, and drugs that would sensitize HER2-targeting agents toward apoptosis could further increase their efficacy in the clinic.

Rapamycin and its derivatives have been introduced into several clinical trials in the past couple of years. Rapamycin inhibits the function of mTORC1 complex and leads to the inhibition of the translation through suppressing the 4E-BP1 family of proteins and S6 protein kinases 1 and 2 (41). Interest in the regulation of mTOR has increased substantially in recent years largely because of an apparent link between deregulation of translation and cancer cell survival (42). Many reports have shown the increased cell death with therapeutic agents in combination with inhibitors of mTOR survival pathway (24, 31, 43, 44), suggesting that the mTOR pathway plays an important role in the susceptibilities of chemopreventive or chemotherapeutic agents to kill cancer cells. In accordance with this concept, we showed that luteolin was additive with rapamycin in inhibiting the growth of HER2-overexpressing cancer cells (Supplementary Fig. S4),[3] confirming the importance of inhibiting survival pathways in cancer and indicating that the combination of mTOR inhibitor and HER2-targeted therapies may be a promising strategy for the prevention of disease relapse in patients with HER2-overexpressing breast and ovarian cancers, cancers known to have a poor prognosis, with the potential to maximize efficacy while minimizing toxicity.

Inhibition of Akt/mTOR signaling could be efficiently induced in the treatment of high doses of luteolin but had much lower durability to do so in the treatment of low doses of luteolin (Fig. 4). This is consistent with a recent report by Sergina et al. (45), which finds that, when HER2 is partially blocked by kinase inhibitors, a feedback mechanism renders low doses of kinase inhibitors that are unable to induce sustained inhibition of Akt signaling in HER2-overexpressing cancer cells, thus limiting their antitumor activity. Although the role of p21 in the regulation of apoptosis is conflicting (23), our findings here indicated that the inability to cause sustained inhibition of Akt/mTOR signalings may contribute to cancer cell survival in part through the induction of p21 expression, falling in line with the concept of p21 mediating the survival function in HER2-overexpressing tumors, and further supported the role of p21 as an important determinant of anticancer drug sensitivity. Recent data also showed that overexpression of HER2 in breast cancer cells prevents Taxol-induced apoptosis by transcriptional up-regulation of p21 (29). In fact, a worse disease-free survival was observed in breast cancer patients treated with adjuvant chemotherapy regimens containing cyclophosphamide, methotrexate, and 5-fluorouracil when the tumors expressed a high level of p21 and HER2, suggesting that p21 may play a role in HER2-mediated cyclophosphamide, methotrexate, and 5-fluorouracil resistance (46). Thus, the increase of p21 seen in HER2-overexpressing cancers cells may give these cells certain survival advantages.

Because p21 protects cells from anticancer drug-induced apoptosis and that disabling apoptotic responses might be a major contributor to drug resistance, it raises concerns about how cancer cells can bypass a given selection pressure through up-regulation of an alternative signaling. Given that MDA-MB-453 cells express mutant p53 protein and that SKOV3.ip1 cells are p53 null, however, it is reasonable to deduce that the increased p21 level is through a p53-independent mechanism. It has been shown that inhibition of Rho GTPases contributes to p21 elevation through both increased transcription and protein stabilization (47). Notably, IκB kinase β overexpression is able to up-regulate Akt phosphorylation and its expression is highly associated with the expression of total and cytoplasmic p21 in primary breast cancers (48). The cytoplasmic localization of p21 has been proposed to be critical in promoting HER2-overexpressing cancer cell survival (49), which is consistent with our findings that the up-regulated p21 is mainly in the cytoplasm (Fig. 7B, *g*). Currently, the molecular mechanism of the induced p21 expression by low doses of luteolin is still under investigation.

Several lines of evidence suggest that Hsp90 molecular chaperone is involved in the HER2 depletion effect of luteolin. First, luteolin induced dissociation of Hsp90-HER2 complex (Fig. 1E). Second, similar to geldanamycin-induced HER2 degradation, proteasomal activity was involved in luteolin-induced HER2 degradation (Fig. 1D), suggesting that the degradation of HER2 was a necessary step in HER2 depletion in cells treated with luteolin. Third, molecular modeling showed that luteolin has a high affinity with Hsp90, suggesting that luteolin could bind to Hsp90 and interfere with its association with HER2 (data not shown). Fourth, besides HER2, other Hsp90 client proteins, such as Akt and cyclin-dependent kinase 4, were also reduced by luteolin (Fig. 4; Supplementary S5).[3] Furthermore, our unpublished data indicated that luteolin was also able to promote androgen receptor degradation in androgen-dependent LNCaP cells.[4] Overall, these results suggest that luteolin diminishes the interaction of HER2 with Hsp90, which in turn leads to a degradation of HER2 protein.

Taken together, the central and novel findings in the present study are that (*a*) luteolin decreases the expression

[4] In preparation.

level of HER2 in HER2-overexpressing cancer cells both *in vitro* and *in vivo*; (*b*) luteolin significantly suppresses the growth of HER2-overexpressing cancer cells both *in vitro* and in tumor xenografts in nude mice; and (*c*) low doses of luteolin induce p21 expression, and the inhibition of p21 expression by mTOR inhibitor rapamycin or p21 siRNA results in increased sensitivities to luteolin-induced apoptosis. These findings may help increase the efficacy of preventive or therapeutic compounds against HER2-overexpressing cancer cells.

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Sensitizing HER2-overexpressing cancer cells to luteolin-induced apoptosis through suppressing p21 WAF1/CIP1 expression with rapamycin

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