Anticancer effect of luteolin is mediated by downregulation of TAM receptor tyrosine kinases, but not interleukin-8, in non-small cell lung cancer cells

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Abstract. TAM receptor tyrosine kinases (RTKs), Tyro3, Axl and MerTK, transduce diverse signals responsible for cell survival, growth, proliferation and anti-apoptosis. In the present study, we demonstrated the effect of luteolin, a flavonoid with antioxidant, anti-inflammatory and anticancer activities, on the expression and activation of TAM RTKs and the association with its cytotoxicity in non-small cell lung cancer (NSCLC) cells. We observed the cytotoxic effect of luteolin in parental A549 and H460 cells as well as in cisplatin-resistant A549/CisR and H460/CisR cells. Exposure of these cells to luteolin also resulted in a dose-dependent decrease in clonogenic ability. Next, luteolin was found to decrease the protein levels of all three TAM RTKs in the A549 and A549/CisR cells in a dose-dependent manner. In a similar manner, in H460 and H460/CisR cells, the protein levels of Axl and Tyro3 were decreased following luteolin treatment. In addition, Axl promoter activity was decreased by luteolin, indicating that luteolin suppresses Axl expression at the transcriptional level. We next found that luteolin abrogated Axl phosphorylation in response to growth arrest-specific 6 (Gas6), its ligand, implying the inhibitory effect of luteolin on Gas6-induced Axl activation. Ectopic expression of Axl was observed to attenuate the antiproliferative effect of luteolin, while knockdown of the Axl protein level using a gold nanoparticle-assisted gene delivery system increased its cytotoxicity. In contrast to the inhibitory effect of luteolin on the expression of TAM RTKs, interleukin-8 (IL-8) production was not decreased by luteolin in H460 and H460/CisR cells, while IL-8 production/cell was increased. Collectively, our data suggest that TAM RTKs, but not IL-8, are promising therapeutic targets of luteolin to abrogate cell proliferation and to overcome chemoresistance in NSCLC cells.

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Abbreviations: AuNP, gold nanoparticle; IL-8, interleukin-8; Gas6, growth arrest-specific 6; GDS, gene delivery system; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase

Key words: Axl, Gas6, IL-8, luteolin, RTK, TAM

Introduction

Platinum-based chemotherapy including cisplatin is the first-line therapy for the treatment of NSCLC (1). However, NSCLC remains the leading cause of cancer-related deaths which are attributed to late diagnosis and development of chemoresistance. Many mechanisms and several molecules involved in chemoresistance have been investigated, but therapeutic outcomes are still unsatisfactory.

The TAM receptor tyrosine kinase (RTK) family, consisting of Tyro3, Axl, MerTK is a subfamily of RTK, which has been reported to be involved in cell growth, proliferation, metastasis and resistance to chemotherapy (2) in various types of cancers. TAM RTKs also play important roles in immune regulation, since they function as phagocytic receptors in normal tissues (3,4). In addition, structural features of TAM RTKs in the extracellular, transmembrane and cytoplasmic kinase domains are very similar (3), which allow them to share various ligands including growth arrest-specific 6 (Gas6), protein S, tubby and tulip. Gas6 has been known to bind to all three TAM RTKs with higher affinity for Axl than the others (5). Upon activation of Axl by Gas6 binding, tyrosine residues of the cytoplasmic tyrosine kinase domain are auto-phosphorylated (6), which in turn activate several signaling pathways mediating cell growth, survival, proliferation, migration and inhibition of apoptosis (3,7).

Axl, also referred to Ark, Tyro7 or Ufo, was first isolated from chronic myelogenous leukemia cells (8). Since then, Axl overexpression has been reported in a multitude of cancers

including acute leukemia (9), breast (10), colon (11), esophageal, lung (12), ovarian (13), prostate (14) and thyroid cancer (15). The overexpression of Axl has been shown to be associated with epithelial-to-mesenchyaml transition (EMT), anticancer drug resistance and angiogenesis (16-18), whereas Axl inhibition decreased cancer cell growth and migration and increased sensitivity to anticancer drugs (19). The overexpression of Axl is observed in ~50% of clinical specimens of lung cancer cases and 60% of NSCLC cell lines and is associated with increased invasion and poor prognosis (20,21). Moreover, Axl expression mediates resistance to anti-EGF receptor (EGFR) therapy including gefitinib and erlotinib in NSCLC (22,23). The inhibition of Axl with Axl-specific siRNA or monoclonal antibodies has been reported to decrease the proliferation of NSCLC cells *in vitro* and *in vivo* in a tumor xenograft model (24,25) and depletion of Rac1, a downstream effector of Axl has been shown to result in enhanced sensitivity to anticancer drugs (26,27).

Interleukin (IL)-8 is a proinflammatory cytokine overexpressed in many types of cancer including colon carcinoma, melanoma (28), ovarian (29) and prostate cancer (30), and several studies recently demonstrated that upregulation of IL-8 expression was associated with the acquired resistance against various chemotherapeutic drugs such as cisplatin (31,32), paclitaxel (33), and a receptor tyrosine kinase (RTK) inhibitor, erlotinib which specifically targets epidermal growth factor receptor (EGFR), in ovarian (33), lung (34,35) and head and neck cancer (HNC) cells (36), respectively. Particularly, in erlotinib-resistant HNC cells, the expression of a panel of genes including IL-8, EGFR and Axl was found to be increased (36). In addition, Gong *et al* reported that in gastric cancer, ErbB2, a member of the EGFR family, is activated and that an ErbB2-targeting agent, trastuzumab, decreased the expression of IL-8 (37). Therefore, IL-8 has received a lot of attention as a potent therapeutic target to control cancer progression as well as chemoresistance and it appears to be quite beneficial to examine whether auto- and/or paracrine regulation of IL-8 production is associated with the expression of some RTKs such as EGFR and Axl or vice versa.

Luteolin, 3',4',5,7-tetrahydroxyflavone, is a non-toxic flavonoid widely found in various plants and has many biological activities including antioxidant, anti-inflammatory and anticancer effects. Recent studies have shown that the luteolin induced sensitization of many different cancers to therapeutic drugs (38-40), and its anticancer effects were mediated by diverse signaling pathways involved in cell proliferation, angiogenesis, metastasis and apoptosis (41). However, the effect of luteolin has not been studied yet in the expression and activation of TAM RTKs and the association with its cytotoxicity.

In the present study, we tested the association of TAM RTKs in the anticancer effect of luteolin in parental and cisplatin-resistant NSCLC cells to provide a potent therapeutic target to inhibit cell proliferation and overcome chemoresistance.

Materials and methods

Reagents and antibodies. Luteolin was obtained from Sigma-Aldrich (St. Louis, MO, USA). A549 and H460 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both control shRNA and Axl shRNA which were annealed to gold nanoparticles were synthesized by the domestic company, Bioneer Corp. (Daejeon, Korea). Lipofectamine 2000 and G418 were obtained from Roche Diagnostics Corp. (Indianapolis, IN, USA) and Gibco-BRL (Gaithersburg, MD, USA), respectively. The plasmid, pGL3-basic vector, and the Dual-Glo luciferase assay kit were purchased from Promega Corp. (Madison, WI, USA). For western blot analysis, specific antibodies against Axl, phosphor-Axl, MerTK, Tyro3 and GAPDH, as well as secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture and establishment of cisplatin-resistant cells. The A549 and H460 cells were grown in RPMI-1640 medium (Gibco-BRL) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/ml penicillin and 10 g/ml streptomycin at 37˚C in 5% CO_2 in a water-saturated atmosphere. Cisplatin-resistant cells, A549/CisR and H460/CisR, were established by stepwise exposure of parental cells to escalating concentrations of cisplatin (ranging from 0.5 to 2 μM).

Reverse transcription PCR (RT-PCR). Cells ($3x10^5$) were seeded in a 60 mm culture dish and grown overnight. They were then treated with the indicated concentrations of luteolin for 24 h. Total RNA was extracted using TRI reagent and subjected to cDNA synthesis and PCR. The specific primers were as follows: Axl sense, 5'-AACCTTCAACTCCTGCCTT CTCG-3' and antisense, 5'-CAGCTTCTCCTTCAGCTCTTC AC-3'; GAPDH sense, 5'-GGAGCCAAAAGGGTCATCAT-3' and antisense, 5'-GTGATGGCATGGACTGTGGT-3'.

Promoter activity assessment. The promoter reporter plasmid, pGL3-Axl, which contains the *Axl* promoter region ranging from -887 to +7 bp of the transcriptional start site was amplified by PCR and subcloned into the pGL3-basic vector, the luciferase reporter plasmid. The constructed promoter-reporter plasmid was co-transfected into cells ($3x10^5$ cells in a 60-mm dish) with *Renilla* luciferase vectors, pRL-SV40, as an internal control. Luciferase activity was asessed using a Dual-Glo luciferase assay system.

Western blot analysis. Total cell lysates were prepared from the parental or chemoresistant cells treated with the indicated concentrations (0, 20, 40 and 80 μM) of luteolin using lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM Na_3VO_4 and protease inhibitor cocktail]. Untreated cells were used as controls. Protein concentrations were determined using Bio-Rad protein assays. Proteins from the cell lysates (20-40 μg) were separated by 12% SDS-PAGE, and electrotransferred onto nitrocellulose membranes. The membranes were blocked for 30 min at room temperature in Tris-buffered saline with 0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary antibody for 4 h at room temperature. After 3x10 min washes in TTBS, the membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h. Following three additional 10-min washes with TTBS, the protein bands of interest were visualized using an enhanced chemiluminescence detection

system (Amersham™ ECL™ Prime Western Blotting Detection Reagent; GE Healthcare, Piscataway, NJ, USA). The density of each protein level was measured by LAS-3000 FujiFilm Image Reader and Multi-Gauge 3.0 software and the Axl protein level was normalized with that of GAPDH.

Cell viability measurement. To assess cell viability, the number of viable cells was counted after trypan blue staining. Briefly, $3x10^3$ cells were seeded into a 60-mm culture dish, grown overnight and then treated with the indicated concentrations (0, 20, 40 and 80 μM) of luteolin for 24 h. After luteolin treatment, cells were harvested and stained with 0.4% trypan blue solution. Dye-excluding viable cells were counted under the microscope. Cell viability was also expressed as a percentage of the viable cells with respect to the untreated control cells. Additionally, the viability of cells was assessed using Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Laboratories, Kumamoto, Japan). Cells ($1x10^3$ cells/well) were seeded in 96-well plates and grown overnight, and then treated with the indicated concentrations of luteolin with or without 4 μM cisplatin for the 24 h. At the end of the treatment, 10 μl of CCK-8 solution was added and further incubated for 4 h. The absorbance at 450 nm was measured using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories, Hercules, CA, USA). Values are expressed as the mean ± SD for triplicate wells and normalized to that of the control group to determine the % of viability.

Colony formation assay. Cells were seeded into 24-well plates ($1x10^2$ cells/well) and treated with the indicated concentrations (0, 20, 40 and 80 μM) of luteolin for 24 h. Luteolin-treated cells were then cultured for the next 7-10 days to form colonies. Colonies of >50 cells were stained with crystal violet (in 60% methanol; Junsei Chemical Co., Ltd., Tokyo, Japan) and images were acquired using the RAS-3000 Image Analysis System (FujiFilm, Tokyo, Japan).

Ectopic expression of Axl. To ectopically express Axl, the recombinant plasmid, pcDNA3-Axl, was constructed by cloning the Axl cDNA into the *Eco*RI and *Bam*HI sites of the pcDNA3 vector and 2 μg of purified plasmids were transfected into the A549 or A549/Cis cells ($3x10^5$ cells in a 100-mm dish) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To establish stable cell lines, which constitutively express Axl, the transfected cells were cultured in the presence of 400 μg/ml of G418. The RPMI-1640 medium containing G418 was refreshed every three days. After three to four weeks, the Axl-expressing cells were enriched and the Axl expression in these cells was analyzed by western blot analysis.

Gold nanoparticle-assisted gene delivery system for Axl silencing. Chemically functionalized gold nanoparticles (AuNPs) were annealed with the shRNA targeting *Axl* gene (5'-TAATACGACTCACTATAGGGAAGAUUUGGAGAACACACUGA-3') and used as a gene delivery system (GDS) to decrease Axl expression. Briefly, cells ($3x10^5$) were seeded in 60-mm culture dishes, grown overnight and then incubated with 10 nM AuNPs-*Axl* or control AuNPs. The cells were harvested for 24 and 48 h after transfection and used to evaluate protein expression and cell proliferation, respectively.

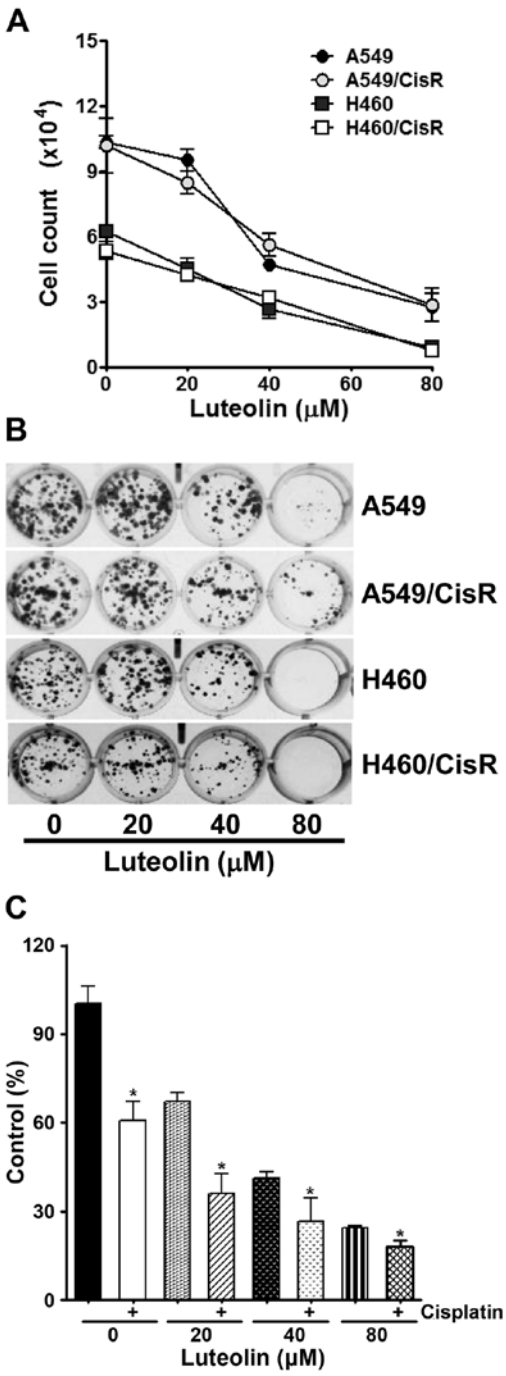

Figure 1. Luteolin inhibits cell proliferation of parental and cisplatin-resistant non-small lung cancer cells. (A) A549, H460 and their cisplatin-resistant cells (A549/CisR and H460/CisR) were seeded into 60 mm dishes ($3x10^5$ cells/dish) and grown overnight. Cells were treated with 20, 40 and 80 μM of luteolin for 24 h, and then cells were harvested and stained with trypan blue. The number of viable cells was counted. Data are expressed as the means ± SD of triplicate samples conducted in three independent experiments. The asterisks indicate the significant difference compared to the control value ($^*P<0.05$, vs. the untreated group). (B) Cells ($2x10^3$ cells/well) were seeded into a 24-well culture plate, grown overnight, exposed to 20, 40 and 80 μM of luteolin, and allowed to grow for the next 7-10 days. The colonies were visualized by crystal violet staining. The data shown are representative of at least three independent experiments. (C) H460/CisR cells ($1x10^3$ cells/well) were seeded in 96-well plates, grown overnight and then treated with the indicated concentrations of luteolin in the absence or presence of 4 μM cisplatin for the 24 h. To assess the cell viability, CCK-8 solution was added into each well and the absorbance at 450 nm was assessed. Values are normalized by the absorbance of the control group and expressed as the mean ± SD for triplicate wells. The asterisks indicate the significant difference compared to the value of cells without cisplatin treatment ($^*P<0.05$).

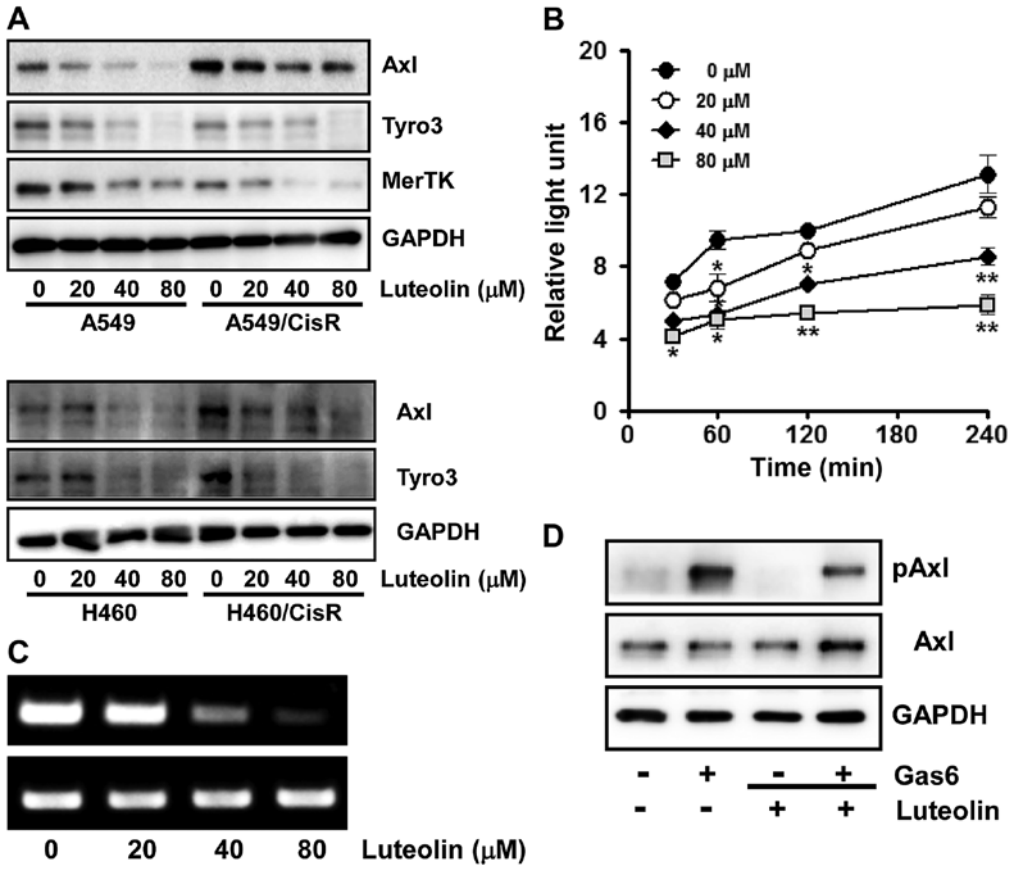

Figure 2. Luteolin downregulates the expression of TAM RTKs and inhibits Axl activation upon Gas6 stimulation in non-small lung cancer cells. (A) A549, H460, A549/CisR and H460/CisR cells ($3x10^5$ cells/dish) were seeded onto 60-mm dishes, grown overnight, treated with 20, 40 and 80 μM of luteolin for 24 h, and then harvested. The total cell lysates were prepared to determine Tyro3, Axl and MerTK protein levels by western blot analysis. GAPDH was used as a loading control and the results shown are a representative of at least three independent experiments. (B) For RT-PCR, H460 cells ($3x10^5$ cells) were seeded into 60 mm culture dishes, grown overnight and treated with the indicated concentrations of luteolin for 24 h. Total RNAs from the cells were isolated and used to determine Axl mRNA levels. GAPDH mRNA was also amplified by RT-PCR as an internal control. The data shown are a representative of three independent experiments The asterisks indicate a significant difference compared to the control value ($^*P<0.05$, $^{**}P<0.01$, vs. the untreated group). (C) To assess the effect of luteolin on Axl promoter activity, the H460 cells ($3x10^4$ cells) were transfected with pGL3-Axl using Lipofectamine 2000. The cells were then incubated with 20, 40 and 80 μM of luteolin for 30, 60, 120 and 240 min and total cell lysates were used to measure luciferase activity. Data are expressed as the means ± SD of triplicate samples conducted in three independent experiments. (D) H460 cells ($3x10^5$ cells/dish) were pre-incubated with 40 μM of luteolin for 60 min and stimulated with Gas6 (250 ng/ml) for 20 min. Phospho- and total Axl protein levels were determined by western blot analysis. Total Axl protein level was used as a loading control. Results shown are representative of three independent experiments.

Statistical analysis. Data are expressed as the means ± SD of triplicate samples or at least three independent experiments. To determine statistical significance, the Student's t-test was used with a P-value threshold of <0.05.

Results

Luteolin inhibits proliferation of both parental and cisplatin-resistant non-small lung cancer cells. The antiproliferative effect of luteolin was first examined in parental non-small cell lung cancer (NSCLC) A549 and H460 cells, as well as cisplatin-resistant A549/CisR and H460/CisR cells. As shown in Fig. 1A, the viability of these cells was found to be decreased in a dose-dependent manner. Next, a clonogenic assay was further performed to confirm the cytotoxicity of luteolin in A549, H460, A549/CisR and H460/CisR cells. These cells were treated with the indicated concentrations of luteolin, and then allowed to grow for the next 10 days. Luteolin treatment resulted in the dose-dependent decrease in the colony formation (Fig. 1B). Notably, H460 and H460/CisR cells that were incubated with 80 μM luteolin failed to form visible colonies, suggesting that luteolin seems to be more cytotoxic to H460 cells than to A549 cells. We also examined the effect of co-treatment of luteolin and cisplatin on cell proliferation using H460/CisR cells. While 4 μM cisplatin alone decreased the viability of the H460/CisR cells to 61% and luteolin decreased that to 67, 41 and 27% in proportion to the concentration of luteolin, co-treatment of cells with luteolin and cisplatin reduced that to 36, 27 and 18%, respectively (Fig. 1C). The results demonstrated that in the presence of cisplatin, the cytotoxicity of luteolin was additively increased in the cisplatin-resistant cells.

Luteolin downregulates the expression of RTKs and inhibits Axl activation upon its ligand binding. Since TAM RTKs have been reported to be associated with oncogenesis, proliferation, survival and anti-apoptosis (9,10,42,43), we next assessed the effect of luteolin on the expression of TAM RTKs. Cells were incubated with the indicated concentrations of luteolin for 24 h and the protein levels of each TAM RTK was

measured by western blot analysis. We found that in the A549 and A549/CisR cells, luteolin treatment dose-dependently decreased the expression levels of all three TAM RTKs, Tyro3, Axl and MerTK. Similarly in the H460 and H460/CisR cells, the expression levels of both Axl and Tyro3 were decreased by luteolin (Fig. 2A). Notably, in the H460 cells, MerTK was undetectable by western blot analysis.

Consistent with the western blot results, the inhibitory effect of luteolin on the *Axl* gene expression was further demonstrated by RT-PCR and assessment of *Axl* promoter activity. As shown in Fig. 2B, the mRNA level of Axl was decreased by luteolin treatment in a dose-dependent manner. Additionally, H460 cells transfected with pGL3-Axl, the *Axl* promoter-luciferase reporter plasmid, were treated with 0, 20, 40 and 80 μM of luteolin for the indicated time periods and luciferase activity was found to be dose-dependently decreased by luteolin treatment (Fig. 2C), indicating that luteolin suppresses Axl expression at the transcriptional level.

Since growth arrest-specific gene 6 (Gas6) binds to all three TAM RTKs and is highly specific to Axl (5), we next observed the effect of luteolin on Axl activation upon ligand binding. Serum-starved H460 cells were pre-treated with luteolin and then stimulated with Gas6. As illustrated in Fig. 2D, Gas6-induced Axl phosphorylation was significantly inhibited by luteolin, indicating that luteolin suppresses tyrosine kinase activity of Axl which is mediated by autophosphorylation of tyrosine residues in the intracellular kinase domain in response to ligand binding (6).

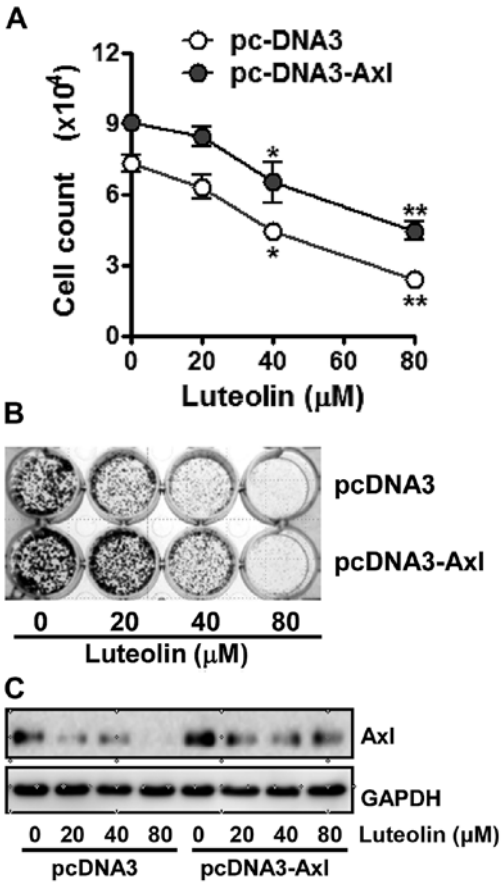

Figure 3. Antiproliferative effect of luteolin is decreased by Axl overexpression in non-small lung cancer cells. H460 cells were transfected with pcDNA3 or pcDNA3-Axl plasmid using Lipofectamine 2000. (A) The transfected cells ($3x10^5$ cells/dish) were treated with 20, 40 and 80 μM of luteolin for 24 h, then harvested, and stained with trypan blue in order to count the viable cells. Data are expressed as the means ± SD from three independent experiments. The asterisks indicate a significant difference compared to the control value ($^*P<0.05$, $^{**}P<0.01$, vs. the untreated group). (B) Cells ($2x10^3$ cells/well) transfected with pcDNA3 or pcDNA3-Axl plasmid were seeded into 24-well culture plates, grown overnight, exposed to 20, 40 and 80 μM of luteolin, and allowed to grow for the next 7-10 days. The colonies were visualized by crystal violet staining. The data shown are representative of at least three independent experiments. (C) The transfected cells ($3x10^5$ cells/dish) were treated with 20, 40 and 80 μM of luteolin for 24 h, then harvested, and Axl protein levels were determined by western blot analysis. GAPDH was used as a loading control and results shown are representative of at least three independent experiments.

Luteolin affects the expression of Axl, but not IL-8, to exert its antiproliferative effect. To validate the association of Axl in the antiproliferative effects of luteolin, we examined its cytotoxicity toward the H460 cells in which the Axl protein was overexpressed or knocked down, respectively. As shown in Fig. 3A, cells which were transfected with pcDNA3-Axl containing *Axl* cDNA for ectopic expression of Axl were found to be a less sensitive to luteolin than cells transfected with the pcDNA3 vector (Fig. 3A). Colony formation assay results also showed that clonogenicity of Axl-overexpressing cells was less affected by luteolin treatment compared with the cells transfected with pcDNA3, since luteolin treatment led H460/pc-DNA3 cells to form less colonies and the size of each colony was smaller than that of the H460/pc-DNA3-*Axl* cells (Fig. 3B). Consistent with the cell viability and colony formation assays, the Axl protein level in the H460/pcDNA3-*Axl* cells was found to be higher than that in the control cells even after luteolin treatment (Fig. 3C). These results point to the fact that overexpression of Axl attenuates the antiproliferative effect of luteolin.

Next, we examined whether knockdown of *Axl* increases the antiproliferative effect of luteolin. To decrease Axl expression, we used gold nanoparticle (AuNP)-assisted gene delivery systems (GDS) (44,45). H460 cells were incubated with AuNP GDS conjugates which were annealed with Axl-specific shRNA, or control shRNA. As shown in Fig. 4A, AuNP-GDS-*Axl* conjugates resulted in the additional decrease in Axl expression following luteolin treatment. The cell viability assay also showed that the AuNP-GDS-*Axl* conjugates increased cytotoxicity of luteolin (Fig. 4B). These results indicate that the amount of Axl protein was tightly correlated with cell viability and imply that luteolin exerts an antiproliferative effect via the downregulation of Axl expression. Collectively, these results indicate that luteolin inhibits Axl expression, concomitantly induces the protein level of p21, and subsequently abrogates cell proliferation.

Since IL-8, a multifunctional inflammatory cytokine, has been reported to be associated with cell survival, growth, angiogenesis and metastasis in various types of cancers such as melanoma, lung cancer, nasopharyngeal, hepatocellular, ovarian, colorectal and prostate cancer (33,46-49), we observed the effect of luteolin on IL-8 expression. ELISA results showed that the IL-8 production in cells treated with 80 μM luteolin was decreased, whereas 20 and 40 μM luteolin rather increased IL-8 level (Fig. 5A). Notably, IL-8 expression/cell was found to be fairly increased by luteolin treatment (Fig. 5B), indicating

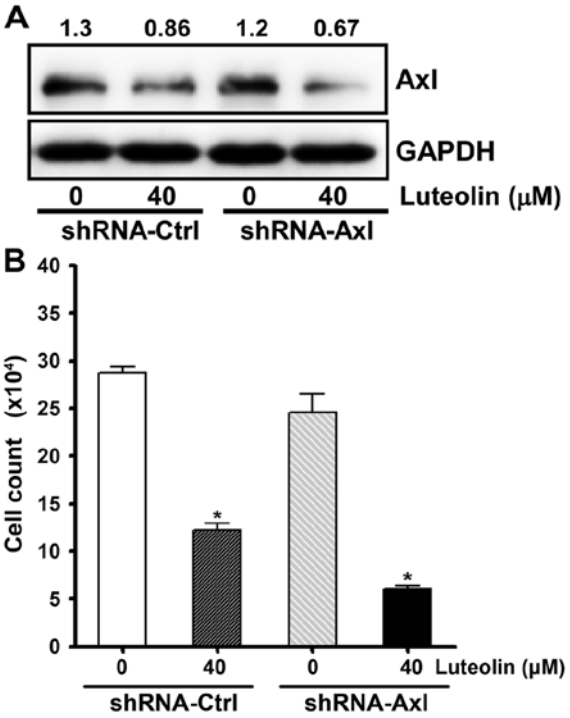

Figure 4. Knockdown of Axl expression increases the antiproliferative effect of luteolin and attenuates Axl induction in non-small lung cancer cells. H460 cells were treated with gold nanoparticle-control shRNA or Axl-specific shRNA. The gold nanoparticle-treated cells ($3x10^5$ cells/dish) were incubated with 40 μM of luteolin for 24 h and then harvested. (A) The protein levels of Axl were determined by western blot analysis. GAPDH was used as a loading control and the results shown are representative of at least three independent experiments. (B) Cells were stained with trypan blue in order to count the viable cells. Data are expressed as the means ± SD from three independent experiments. The asterisks indicate a significant difference compared to the control value (*P<0.05, vs. the untreated group).

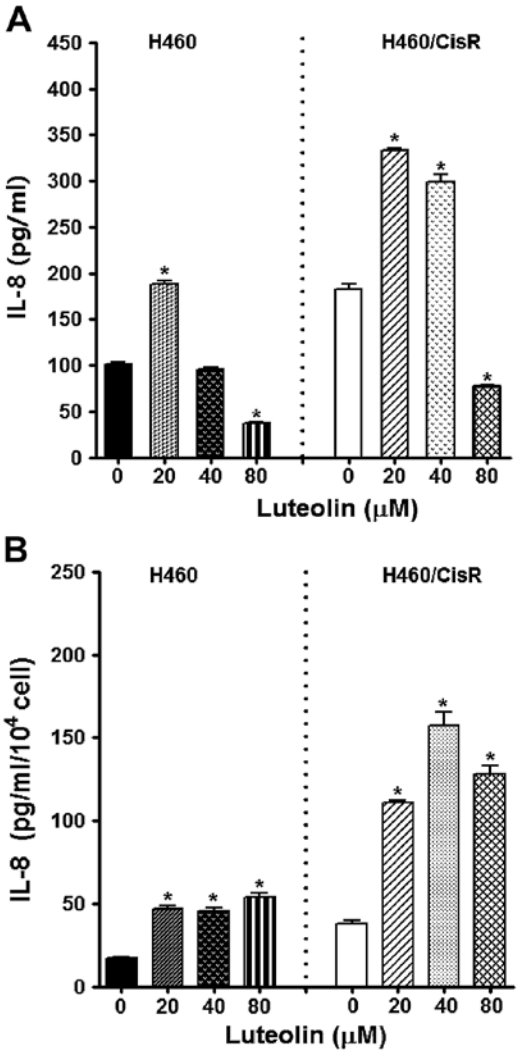

Figure 5. IL-8 expression is not affected by luteolin in both parental and cisplatin-resistant non-small lung cancer cells. H460 and H460/CisR cells ($3x10^5$ cells/dish) were seeded onto 60-mm dishes, grown overnight and treated with 20, 40 and 80 μM of luteolin for 24 h. (A) Culture media were harvested and used for IL-8 ELISA. Data are expressed as the mean ± SD of triplicate samples. The asterisks indicate a significant difference compared to the control value (*P<0.05 vs. the untreated group). The results are representative of at least three independent experiments. (B) To determine IL-8 production/cell, the total amount of IL-8 was normalized by the number of viable cells. Data are expressed as the mean ± SD of triplicate samples. The asterisks indicate a significant difference compared to the control value (*P<0.05 vs. the untreated group).

that the antiproliferative effect of luteolin was not associated with dysregulation of IL-8 expression.

Discussion

A series of recent studies have shown the positive correlation between the expression levels of Axl and the resistance to anticancer drugs, invasiveness and poor outcome in several types of cancer. Moreover, Axl silencing was found to significantly reduce the cell proliferation in NSCLC cells. Therefore, Axl has received more and more attention as a promising therapeutic target in the restriction of disease (50).

In the present study, luteolin was found to suppress expression of all three TAM receptor tyrosine kinases (RTKs) (Fig. 2A). We also observed the inhibitory effect of luteolin on Axl expression (Fig. 2B and C) and Gas6-induced activation of Axl (Fig. 2D). In addition, our data demonstrated that overexpression of Axl by ectopic expression of the *Axl* gene or knockdown of the Axl protein levels by gold nanoparticle-assisted gene delivery system led to the attenuation or increase of luteolin-induced cytotoxicity, respectively (Figs. 3A and B, and 4B). These results strongly indicate that luteolin targets TAM RTKs, particularly Axl, to function as an anticancer drug. Notably, some increase in the protein level of Axl was found in cisplatin-resistant A549/CisR and H460/CisR cells (Fig. 2A), which appears to be a strategy in order to survive in the presence of cisplatin and/or a mechanism for the acquisition of cisplatin-resistance. However, cisplatin-resistant cells were still sensitive enough to luteolin in spite of the elevated Axl protein level of these cells, suggesting that luteolin can potentially overcome chemoresistance and that there must be other molecules including Tyro3 and MerTK which are affected by luteolin and contribute to its cytotoxicity.

Increasing evidence indicates that cancer-related inflammation of which cytokines are key constituents promotes survival and proliferation of malignant cells (47), supports angiogenesis and metastasis (51-53), and evokes epithelial-to-mesenchymal transition (EMT) (54,55) and chemoresistance against anticancer agents (33,56,57). Interleukin-8 (IL-8) has been

reported to be overexpressed in tumor tissue and associated with advanced stage disease and poor prognosis (48). Consistent with previous studies (48,58), we found that the level of IL-8 was increased in the cisplatin-resistant H460/CisR cells compared to the parental H460 cells. However, luteolin did not appear to be useful in modulating the levels of IL-8, since only a high concentration of luteolin (80 μM) was found to decrease the expression IL-8 and relatively low concentrations of luteolin (20 and 40 μM) were observed to elevate the levels of IL-8. In parallel, we also found that IL-8 production from a single cell was increased by luteolin. Our results indicate that an adjuvant therapy which decreases the levels of IL-8 or blocks IL-8-mediated signaling pathways may result in a synergistic outcome in cancer treatment. Therefore, IL-8 is a noteworthy target for improved cancer treatment.

In summary, we demonstrated that luteolin suppressed the expression of TAM RTKs, but not IL-8, and activation of Axl upon Gas6 binding, facilitated the inhibitory effect of luteolin on cell proliferation in both the parental and cisplatin-resistant NSCLC cells. Thus, our data imply that TAM RTKs may be potent therapeutic targets of luteolin by which it exerts its anti-cancer activity, particularly to circumvent chemoresistance in NSCLC cells.

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