Sulfasalazine Suppresses Drug Resistance and Invasiveness of Lung Adenocarcinoma Cells Expressing AXL

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Abstract
Metastasis and drug resistance are the major causes of mortality in patients with non–small cell lung cancer (NSCLC). Several receptor tyrosine kinases (RTKs), including AXL, are involved in the progression of NSCLC. The AXL/MER/SKY subfamily is involved in cell adhesion, motility, angiogenesis, and signal transduction and may play a significant role in the invasiveness of cancer cells. Notably, no specific inhibitors of AXL have been described. A series of CL1 sublines with progressive invasiveness established from a patient with NSCLC has been identified that positively correlates with AXL expression and resistance to chemotherapeutic drugs. The ectopic overexpression of AXL results in elevated cell invasiveness and drug resistance. Nuclear factor-κB (NF-κB) signaling activity is associated with AXL expression and may play an important role in the enhancement of invasiveness and doxorubicin resistance, as shown by using the NF-κB inhibitor, sulfasalazine, and κB dominant-negative transfectants. In the current study, sulfasalazine exerted a synergistic anticanicancer effect with doxorubicin and suppressed cancer cell invasiveness in parallel in CL1 sublines and various AXL-expressing cancer cell lines. Phosphorylation of AXL and other RTKs (ErbB2 and epidermal growth factor receptor) was abolished by sulfasalazine within 15 min, suggesting that the inhibition of NF-κB and the kinase activity of RTKs are involved in the pharmacologic effects of sulfasalazine. Our study suggests that AXL is involved in NSCLC metastasis and drug resistance and may therefore provide a molecular basis for RTK-targeted therapy using sulfasalazine to enhance the efficacy of chemotherapy in NSCLC. [Cancer Res 2007;67(8):3878–87]

Introduction
Non–small cell lung cancer (NSCLC) is the most common and lethal cancer in the world (1). Development of distant metastasis after tumor resection is the leading cause of death in patients with NSCLC (1, 2). The development of drug resistance in metastatic tumor cells has become a major barrier to the efficacy of chemotherapy (2). Several molecules have been implicated in cancer cell invasiveness and metastasis. Specifically, the receptor tyrosine kinases (RTKs) are involved in tumorigenesis of NSCLC and may thus serve as potential markers for the prognosis and metastatic potential in early-stage NSCLC (3). RTKs convey potent signals that govern various aspects of cell behavior. Indeed, aberrant signal transduction is often involved in the mechanism underlying metastasis (3). In NSCLC, only a few RTKs [e.g., epidermal growth factor receptor (EGFR) and ErbB2] have been thoroughly studied (3–5). AXL/MER/SKY is a new class of the RTK subfamily that transduces crucial extracellular signals inside cells (6). Proteins of this subfamily contain two extracellular fibronectin type III domains (FN3) and two immunoglobulin-like domains (IG), features that suggest an important role in the interaction between the extracellular matrix and/or other cells (6). AXL, also known as UFO, is a type I transmembrane RTK with transforming activity (7, 8). An increased expression of AXL and increased interaction between AXL and Gas6 have been implicated in antiapoptosis, cell adhesion, and chemotaxis (9–11). The expression of AXL is clearly associated with invasiveness and metastasis in various cancer cell types and is implicated in the prognosis of patients with myeloid leukemia (7, 12), metastatic lung cancer (13, 14), renal cell carcinoma (15), prostatic carcinoma (16, 17), breast cancer (18), and gastric cancer (19). Most recently, AXL was shown to affect multiple cellular behaviors in angiogenesis (14, 20), and independently, blockage of AXL expression results in the suppressed growth of cancer cells in a xenograft model (20). The role of Gas6-AXL signaling in tumor growth and invasion is also supported by the suppression of experimental gliomagenesis in vitro and in vivo using a dominant-negative receptor mutant (AXL-DN; ref. 21).

Although some functional aspects of the AXL/MER/SKY family have been deciphered (6), how these receptors mediate their physiologic and pathologic consequences is not well understood. In different cell types, downstream signaling of AXL has been reported to be mediated through tyrosine phosphatase SHP-2 (9), phosphatidylinositol 3-kinase (PI3K)/AKT (10, 22, 23), GTPases of the Rho family (24, 25), glycogen synthase kinase 3 (GSK3; ref. 26), nuclear factor-κB (NF-κB; refs. 11, 27), extracellular signal-regulated kinases (ERK; ref. 25), and p38 mitogen-activated protein kinase (16, 17, 24).

Agents that interfere with AXL and its corresponding signaling pathways may thus provide exciting opportunities for novel therapeutic strategies in a wide range of AXL-associated human malignancies.

Sulfasalazine is a synthetic nonsteroidal anti-inflammatory drug composed of sulfapyridine, a sulfonamide antibiotic, linked via an azo-bond to a 5-aminosalicylic acid moiety (28, 29). Sulfasalazine and related aminosalicylates are commonly used in the management of inflammatory bowel diseases and rheumatoid arthritis (28). The mechanism of action of sulfasalazine requires further study; however, sulfasalazine has been shown to inhibit NF-κB
activation at multiple steps (29–32). Accordingly, small molecular inhibitors of NF-κB, such as sulfasalazine, are likely to provide novel opportunities for improved therapeutic strategies for cancer patients (31, 33, 34).

There is a continuing need for genetically matched cell systems to model cellular behaviors that are frequently observed in aggressive NSCLC. CL-1-0, CL-1-2, CL-1-3, CL-1-4, and CL-1-5 are a series of lung adenocarcinoma cell lines with increasing invasiveness derived from CL-1-0 (35). The more invasive subtype, CL-1-5-F4, was derived from lung metastases of severe combined immunodeficient mice inoculated with CL-1-5 cells (36). AXL is among the many genes associated with the invasiveness of the CL-1 series as determined by microarray studies (36) and further investigated by Shi et al. (14).

In the present study, we have shown that more invasive cells are often more drug resistant and vice versa. Therefore, the CL-1 series of cell lines may serve as a good model for the development of rationale therapies against highly aggressive and therapy-refractory NSCLC. Stable clones of CL-1-0 that ectopically overexpress AXL and stable clones of CL-1-5-F4 with AXL expression knocked down by short hairpin RNA (shRNA)–mediated silencing techniques were established to elucidate the role of AXL in cell invasiveness. Our observations have shown that the reintroduction of AXL into CL-1-0 augments drug resistance and invasiveness and is accompanied by activated NF-κB signaling. By comparison, highly invasive CL-1-5-F4 cells transfected with AXL shRNA vectors diminished NF-κB signaling activities as well as cell invasiveness. The roles of NF-κB signaling in enhanced invasive behavior and drug resistance were shown by using the potent NF-κB inhibitor, sulfasalazine. Disruption of chemoresistance and cell invasiveness by the inhibition of NF-κB activation with sulfasalazine may therefore be a useful new strategy for combination chemotherapies.

Materials and Methods

Cell lines, reagents, and cell viability assay. A CL-1 series of cell lines (i.e., CL-1-0, CL-1-1, CL-1-2, CL-1-3, CL-1-4, CL-1-5, and CL-1-5-F4) was established by selection of increasingly invasive cancer cell populations from a cell line of human lung adenocarcinoma, CL-1-0, using the Transwell invasion chamber assay (35). The following inhibitors of NF-κB signaling were purchased from Calbiochem (San Diego, CA) and dissolved in DMSO (Sigma–Aldrich Chemical Co., St. Louis, MO): 1-phenylalaninecarboxylthioic acid (PDTC), 5-[4-(2-pyridylsulfamoyl) phenylazo]salicylic acid (sulfasalazine), and (E)-3-[(4-methylphenyl) sulfonyl]-2-propenenitrile (Bay11-7082). Each sample was transferred onto a polyvinylidene difluoride membrane, and probed with antibodies against p-ERK1/2 (a mouse monoclonal IgG1; Santa Cruz Biotechnology), ERK1/2 (a rabbit polyclonal IgG; Santa Cruz Biotechnology), phosphorylated AKT (p-AKT, a rabbit polyclonal IgG; Cell Signaling Technology, Inc., Danvers, MA), c-Met (a rabbit polyclonal IgG; Santa Cruz Biotechnology), IκBα (a rabbit polyclonal IgG; Santa Cruz Biotechnology), NF-κB p65 (a rabbit polyclonal IgG; Santa Cruz Biotechnology), phosphorylated Akt (p-AKT; a rabbit polyclonal IgG; Biosource, Carlsbad, CA), phosphorylated ERK (p-ERK; a mouse monoclonal IgG; Santa Cruz Biotechnology), ERK1/2 (a goat polyclonal IgG; Santa Cruz Biotechnology), GSK3α/β (a rabbit polyclonal IgG; Cell Signaling, Waltham, MA), -catenin (a mouse IgG; BD Biosciences), actin (a rabbit polyclonal IgG; Sigma–Aldrich Chemical), and SP-1 (a rabbit polyclonal IgG; Novus Biologicals, Littleton, CO). Actin and SP-1 were used as internal controls for whole-cell and nuclear proteins, respectively. Detection was done with the enhanced chemiluminescence kit (Amersham, Inc., Buckinghamshire, United Kingdom).

In vitro cell migration and invasion assays. Invasiveness of the CL-1 cell lines was examined by using a BioCoat Matrigel invasion chamber system (BD Biosciences, Bedford, MA). The CD44 inhibitor was expressed from Severe Combined Immunodeficient mice inoculated with human glioblastoma cell line DBTRG-6218. The following inhibitors of NF-κB signaling, sequences within the 72- to 86-amino acid NH2-terminal region of IκBα have been reported to be required for inducible IκBα degradation but dispensable for IκBα binding to RelA (p65); therefore, this truncated mutant of human IκBα mutant (p65ΔBD) with amino acids 2 to 71 deleted under cytomagalovirus (CMV) promoter control (parental plasmid was pBK-CMV; Stratagene, La Jolla, CA) was used as a dominant-negative for NF-κB activation in the current study. The NF-κB–driven reporter plasmid (pBEB-Luc) was constructed by linking the luciferase gene to five NF-κB binding sites and a TATA element (37).

Western blot analysis. The levels of RTKs and NF-κB proteins were measured by Western blotting. The methods for preparation of the whole-cell and nuclear proteins have been described previously (38). Each sample equivalent of 50 μg total protein was separated by 8% to 12% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with specific antibodies against AXL (for the COOH terminus of AXL, a goat polyclonal IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; for the NH2 terminus of AXL, MAB154, a mouse monoclonal IgG; R&D Systems, Inc., Minneapolis, MN), MEB (a goat polyclonal IgG; Santa Cruz Biotechnology), SKY (a goat polyclonal IgG; Santa Cruz Biotechnology), GAS6 (a goat polyclonal IgG; Santa Cruz Biotechnology), EGRF (a rabbit polyclonal IgG; Santa Cruz Biotechnology), phosphorylated EGRF (pEGRF), a mouse monoclonal IgG; Invitrogen, Carlsbad, CA), EphA2 (a rabbit polyclonal IgG; Santa Cruz Biotechnology), ErbB2 (AB1369, a rabbit polyclonal IgG; Chemicon International, Inc., Temecula, CA), phosphorylated ErbB2 (p-ErbB2; p Tyr1221/1225), a mouse monoclonal IgG; Cell Signaling Technology, Inc., Danvers, MA), c-Met (a rabbit polyclonal IgG; Santa Cruz Biotechnology), IκBα (a rabbit polyclonal IgG; Santa Cruz Biotechnology), NF-κB p65 (a rabbit polyclonal IgG; Santa Cruz Biotechnology), phosphorylated Akt (p-AKT; a rabbit polyclonal IgG; Biosource, Carlsbad, CA), phosphorylated ERK (p-ERK; a mouse monoclonal IgG; Santa Cruz Biotechnology), ERK1/2 (a goat polyclonal IgG; Santa Cruz Biotechnology), GSK3α/β (a rabbit polyclonal IgG; Cell Signaling, Waltham, MA), -catenin (a mouse IgG; BD Biosciences), actin (a rabbit polyclonal IgG; Sigma–Aldrich Chemical), and SP-1 (a rabbit polyclonal IgG; Novus Biologicals, Littleton, CO). Actin and SP-1 were used as internal controls for whole-cell and nuclear proteins, respectively. Detection was done with the enhanced chemiluminescence kit (Amersham, Inc., Buckinghamshire, United Kingdom).

NF-κB reporter assay. The transcriptional activity of NF-κB was determined by the luciferase reporter assay as described previously (39). The cells were cotransfected with the NF-κB reporter vector (pGL2-luciferase reporter plasmid; Promega Co., Madison, WI) and the
pSV-β-galactosidase construct (Promega) by the LipofectAMINE method (39). The latter construct was used for normalization of transfection efficiency. At the time of harvesting, cell lysates were prepared for assaying luciferase activity according to the LucLite Luciferase Reporter Gene Assay kit (Packard BioScience, Groningen, the Netherlands). All experiments were done in triplicate.

Statistical analysis. The significance of differences in assays conducted in the current study was done with the repeated measures ANOVA or the Student-Newman-Keuls test as indicated.

Results

Increased potential of cell invasion was correlated with drug resistance in CL1 sublines. The morphology and cell invasiveness of the four CL1 sublines are shown in Fig. 1A and C, respectively. Invasiveness through the basement membrane matrix was 2.4-, 4.6-, and 5.3-fold for CL1-1, CL1-5, and CL1-5F4, respectively, over that of the parental CL1-0 cells (P < 0.05, one-way ANOVA). Similar results were also observed in cell migration rates (Supplementary Fig. S1A). Therefore, the differences in migration capacities may also contribute to the progressive invasiveness among these CL1 sublines. Moreover, the anchorage-independent growth on soft agar also showed a similar trend (Supplementary Fig. S1B). The parental CL1-0 cells were larger, rounded, and epithelial-like in the monolayer culture, whereas the more invasive CL1-5F4 cells were more spindle shaped and less adhesive (Fig. 1A).

To characterize the sensitivities of these CL1 sublines to anticancer drugs, 25 traditional chemotherapeutic drugs of different modes of action were screened using the MTT assay.6 CL1 sublines had a gradual increase in their resistance to doxorubicin-induced
cytotoxicity, which paralleled the trends of cell invasiveness (i.e., CL1-0 < CL1-1 < CL1-5 < CL1-5F4; Fig. 1B).

Elevation of AXL in CL1 sublines was correlated with cell invasiveness. Eight proteins (the AXL family, including AXL, MER, and SKY and their common ligand, Gas6, and other RTKs, such as EGFR, ErbB2, EphA2, and MET, implicated to be associated with metastasis of NSCLC) were assayed by Western blot analysis across all four CL1 sublines (Fig. 1D). Among these proteins, only AXL showed a significant increase in parallel with invasiveness. Immunohistochemical staining also showed positive signals of AXL in the cytoplasm and membranes of CL1-5 cells; in comparison, only a very weak signal was observed in CL1-0 cells (Fig. 1A). To further show a correlation between invasion and AXL expression, the Transwell invasion assay was done with CL1-1 cells, and the invaded subpopulation and the noninvaded cells (Supplementary Fig. S2A) were assayed for their AXL transcript by a

Figure 2. Overexpression of AXL increased the migration of CL1-0 cells; silencing AXL expression diminished the migration of CL1-5F4 cells. A, the stable AXL transfectant of CL1-0 (clone 22) with filopodia (arrowhead) on the periphery of the cells compared with blank vector-transfected CL1-0 cells. The AXL-silenced CL1-5F4 transfectant (shAXL2485) was larger, more rounded, and more polygonal-like in shape than the blank vector-transfected CL1-5F4 cells. B, ectopic overexpression of AXL in CL1-0 cells and increased migration. The four clones overexpressing AXL all exhibited increased migration (bottom). Expression of AXL was verified by Western blot (top). C, silencing AXL expression in CL1-5F4 cells and reduction in migration. The four clones with AXL expression knocked down by shRNA all exhibited decreased migration (bottom). Reduced AXL expression was shown by Western blot analysis (top). Columns, mean; bars, SE. ¥, P < 0.05; **, P < 0.01, Student’s paired t test. D, effects of NH2-terminal (N-ter) distal region and COOH-terminal (C-ter) tyrosine kinase domains on cell invasiveness. Top, domains within the 894 residues of the AXL amino acid sequence [IG, FN3, and tyrosine kinase domain (TyrK)]. Representative invasion of CL1-0 transfectants of the five AXL deletion mutants [percentages relative to pAXL-CL1-0 (clone 22; pAXL-CL1-0#22)]. Columns, relative invasion abilities; bars, SE. The invaded cells were counted 24 h after seeding with 5 × 10^4 cells onto the upper chamber of the Matrigel invasion chamber. The expression and molecular weights of various truncated AXL proteins were verified by Western blot analysis against the anti–COOH terminus of AXL [lane 1, CL1-0; lane 2, CL1-5; lane 3, pAXLΔ(33–142); lane 4, pAXLΔ(215–429); lane 5, pAXLΔ(215–326); lane 6, pAXLΔ(333–429); lane 7, pAXLΔ(517–894)] or the anti–NH2 terminus of AXL [lane 8, pAXLΔ(517–894)–transfected CL1-0; lane 9, CL1-5F4]. *, P < 0.05; **, P < 0.01, compared with pAXL-CL1-0 (clone 22) cells.
semiquantitative reverse transcription-PCR. It was found that the AXL transcript of the invaded cells (lower chamber) was ~25-fold higher than that of the noninvaded cells (upper chamber; \( P < 0.01 \), Student’s paired \( t \) test; Supplementary Fig. S2B).

Ectopic overexpression of AXL in CL1-0 cells increased migration ability and filopodia formation, and knockdown of AXL expression in CL1-5F4 cells decreased migration ability. To assess whether AXL potentiates cancer cell invasiveness, CL1-0 cells were transfected with plasmid harboring a full-length human AXL-encoding cDNA. Four stable independent clones were isolated and checked for their status of AXL expression and cell migration abilities (Fig. 2B). The AXL expression levels of clones 16, 18, 14, and 22 were 8-, 63-, 65-, and 62-fold, respectively, and the migration potentials were 2.2-fold, 3.7-fold (\( P < 0.05 \), Student’s paired \( t \) test), 3.9-fold (\( P < 0.05 \), Student’s paired \( t \) test), and 5.1-fold (\( P < 0.01 \), Student’s paired \( t \) test), respectively, when compared with those of the mock-transfected CL1-0 cells. The morphology of clone 22 revealed remarkably increased filopodia formation (Fig. 2A, arrowheads) in the leading edge of the cells compared with the parental CL1-0 cells, where few filopodia were observed (Fig. 2A).

RNA interference is a popular method for silencing gene expression in a variety of systems. In the current study, CL1-5F4 cells were transfected with four shRNA constructs that targeted different regions of the AXL transcripts. The levels of expression of AXL in these stable transfectants were decreased by 90%, 68%, 99%, and 0%, and migration rates decreased by 57% (\( P < 0.05 \), Student’s paired \( t \) test), 34%, 67% (\( P < 0.01 \), Student’s paired \( t \) test), and 5% for shAXL2539, shAXL2185, shAXL2485, and shAXL1882, respectively, compared with those of the mock-transfected CL1-5F4 cells (Fig. 2C). The shAXL2485 transfectant cells appeared to be larger, rounder, and more polygonal shaped than the mock-transfected CL1-5F4 cells, which were smaller and more spindle-like (Fig. 2A).

Ectopic overexpression of AXL and reduced expression of AXL by RNA interference had little effect on cell viabilities and growth rates. Both the NH2-terminal distal region and the COOH-terminal tyrosine kinase domain were important for cell invasiveness. To investigate whether the AXL-related invasiveness of CL1 cells could be mapped to a specific region within the AXL protein, we next constructed a series of truncated AXL mutants and checked their effects on cell invasiveness (Fig. 2D). It seemed that among the four domains tested, the first IG domain of the NH2 terminus was most critical for the role of AXL in invasiveness, as shown with the pAXLΔ(33–142) mutant. The tyrosine kinase domain was also important, as shown with the pAXLΔ(517–894) mutant. In contrast, the two FN3 domains were not as critical, as shown with the two FN3 single-deletion mutants, pAXLΔ(215–326) and pAXLΔ(257–334).
and pAXLΔ(333–429), and the FN3 double-deletion mutant, pAXLΔ(215–429). The levels of expression of the wild-type and various mutants of AXL proteins were relatively comparable as assayed by Western blot analysis (Fig. 2D, bottom).

Overexpression of AXL elevated drug resistance and cells selected for drug resistance showed increased invasiveness. The CL1-0AdrR and CL1-0VP16R sublines were derived from the parental CL1-0 cells by exposure to increasing concentrations of doxorubicin and VP16, respectively. The pAXL-CL1-0 stable transfectants were pooled populations overexpressing AXL. As shown in Fig. 3A, while CL1-0AdrR seemed to be resistant to doxorubicin, as expected and more interestingly, the ectopic overexpression of AXL also conferred resistance to doxorubicin as measured by the MTT assay (Fig. 3A). The doxorubicin IC50 was 0.65 μM/L for pooled pAXL-CL1-0, 3.2 μM/L for CL1-0AdrR, and 0.26 μM/L for the parental CL1-0. The doxorubicin resistance of AXL-overexpressing CL1-0 cells was also shown using a clonogenic assay in the presence of 0.1 μM/L doxorubicin (Fig. 3B). Moreover, drug-resistant CL1-0AdrR and CL1-0VP16R cells both revealed significant morphologic changes, with protruding filopodia in the peripheral cytoplasmic area (Fig. 3C, arrowheads), which is indicative of increased invasiveness. The invasiveness of these drug-resistant sublines was all positively correlated with invasiveness (Fig. 3D, top), and the levels of expression of the AXL protein were all dramatically increased in these drug-resistant cells, as measured by Western blot analysis (Fig. 3D, bottom).

To further evaluate the correlation of AXL levels and chemoresistance in cancer cells, a nonlinear regression analysis was done (Supplementary Fig. S3). The results showed that the AXL levels of expression were positively correlated with the resistance to doxorubicin in 32 human, 2 primate, and 3 rodent cancer or transformed cell lines derived from different tissue types. Consistent with the data shown in Fig. 3, doxorubicin-resistant cancer cells generally expressed higher levels of the AXL protein.

Expression of AXL resulted in NF-κB activation. To elucidate the mechanisms underlying AXL-mediated invasiveness, ERKs, AKT/Pi3K, GSK3, β-catenin, and NF-κB signaling pathways, which have been shown in previous studies (22–27) to be involved in the regulation of AXL-mediated effects, were examined in both CL1-5F4 cells with AXL knocked down by shRNA-mediated silencing and CL1-0 cells ectopically overexpressing AXL. NF-κB activity was assessed by Western blot analysis of nuclear NF-κB p65 and cytoplasmic IκBα (Fig. 4A). Marked accumulation of nuclear NF-κB and degradation of cytoplasmic IκBα were detected in AXL-overexpressing clone 22 and further on addition of the Gas6 ligand. In addition, nuclear NF-κB was diminished and cytoplasmic IκBα was increased in the AXL-knocked down transfectant shAXL2485, suggesting that NF-κB is involved in the signaling pathway of AXL.

Although the levels of p-ERK, p-AKT, GSK3, and β-catenin were much higher in CL1-5F4 than in CL1-0, neither ectopic overexpression of AXL nor knockdown of AXL expression significantly changed the levels of these signal molecules (Supplementary Fig. S4A).

Figure 4. Overexpression of AXL increased invasiveness through the activation of NF-κB. A, analysis of NF-κB signaling pathway proteins. Nuclear (NF-κB p65) and cytoplasmic (IκBα) protein extracts were prepared from shAXL-F4-2485 and pAXL-CL1-0 (clone 22) cells, with or without exogenous Gas6 ligand, for Western blot analysis. Actin and transcription factor SP-1 were used as the loading controls for cytoplasmic and nuclear proteins, respectively. B, NF-κB activity levels in various CL1 sublines and transfectants by the reporter assay. Cells were examined by transient transfection with the NF-κB-driven luciferase reporter in the presence of 10 ng/mL 12-O-tetradecanoylphorbol-13-acetate. Columns, mean; bars, SE. *, P < 0.05; **, P < 0.01, compared with mock vector control, Student’s paired t test. C, suppression of cell invasiveness by dominant-negative IκBα. Stable transfection of the plkBDN vector significantly reduced the cell invasion rate of CL1-5F4 cells. *, P < 0.05, compared with mock vector control, Student’s paired t test. Expression of the dominant-negative IκBα protein was verified by Western blot analysis.
The levels of NF-κB transcriptional activity in the AXL-overexpressing clone 22 and the shAXL2485 transfectant were examined by the NF-κB–driven luciferase reporter assay (Fig. 4B). NF-κB activity of the AXL-overexpressing CL1-0 clone 22 was 2.1-fold (P < 0.05, Student’s paired t test) greater than that of the parental CL1-0 cells, and the NF-κB activity was decreased by 54% (P < 0.01, Student’s paired t test) in the AXL-silenced shAXL2485 transfectant compared with that of the parental CL1-5F4 cells. It seems that the NF-κB activity was correlated with the level of expression of AXL and the metastatic potential of CL1 sublines (Figs. 1B and 4B).

NF-κB signaling pathway was involved in the enhancement of invasiveness of CL1-5F4 cells. To elucidate the underlying signaling mechanism of invasiveness, the effects of several inhibitors of signaling pathways on cell invasion rates were examined in CL1-5F4 cells (Supplementary Fig. S4B). Among the inhibitors tested, only the NF-κB inhibitors sulfasalazine and Bay11-7082 (at IC30 and IC10, respectively) significantly diminished the cell invasion rate of CL1-5F4 cells.

To further elucidate the role of NF-κB signaling, we examined the cell invasion rates of the CL1-5F4 cells and CL1-0 cells transfected with dominant-negative IκBα plasmid (pIkBDN; Fig. 4C). Stable transfection of the pIkBDN vector significantly reduced the cell invasion rate of CL1-5F4 cells (P < 0.05, compared with mock vector control, Student’s paired t test). Expression of the dominant-negative IκBα protein was verified by Western blot analysis (Fig. 4C).

Sulfasalazine dramatically reversed drug resistance and suppressed invasiveness of AXL-expressing cells. Treatment with sulfasalazine and Bay11-7082 at IC30 for 1 day remarkably diminished the cell invasion rate of pAXL-CL1-0 cells ectopically overexpressing AXL (Fig. 5A). Treatment of these cells with sulfasalazine and Bay11-7082 decreased the invasion rates by 78% (P < 0.01, Student’s paired t test) and 74% (P < 0.05, Student’s paired t test), respectively. Because CL1-5F4 cells were the most invasive and most doxorubicin resistant in the CL1 series (Fig. 1B and C), we also tested the effects of combining doxorubicin and various inhibitors of signaling pathways in the CL1-5F4 subline (Fig. 5B). Combination treatment of doxorubicin and sulfasalazine produced...
the most marked effect in inhibiting cell proliferation. Sulfasalazine, at both IC_{10} (12.5 μmol/L) and IC_{30} (33 μmol/L), exerted a significant synergism with doxorubicin in inhibiting cell growth. The synergistic effect was not observed with other agents, such as PDTC, wortmannin, and PD98059. Moreover, sulfasalazine significantly sensitized CL1-0/AdrR cells to doxorubicin (data not shown).

To extend the observation made in CL1-5F4 cells that the cytotoxicity of doxorubicin was potentiated by sulfasalazine, we also showed that sulfasalazine and doxorubicin were a synergistic drug combination in various AXL-expressing cancer cell lines (Supplementary Fig. S5), whereas the combination of doxorubicin and wortmannin did not exhibit a synergistic effect. Importantly, this synergism was not apparent in pI_{jBDN}-CL1-5F4 cells, suggesting that NF-κB signaling plays a crucial role in the chemoresistance of AXL-overexpressing cells (Supplementary Fig. S5).

Sulfasalazine concomitantly inhibited NF-κB activity, AXL phosphorylation, and cell invasiveness. To further clarify the molecular mechanism of action of sulfasalazine and determine its effectiveness as an inhibitor of NF-κB, we specifically examined NF-κB reporter activity of the CL1-5F4 cells treated with sulfasalazine.

The overexpression of many RTKs has been shown to result in receptor phosphorylation and activation (6). We showed herein that endogenously, as well as ectopically AXL-overexpressing sublines, such as CL1-5F4 and pAXL-CL1-0 (clone 22), all expressed elevated levels of phosphorylated AXL (p-AXL; Fig. 6C), and the augmented phosphorylation activities were profoundly inhibited by sulfasalazine by as early as 15 min (Fig. 6C, middle). Interestingly, this inhibition seemed to be NF-κB independent because sulfasalazine sustained this inhibition in the pI_{jBDN}-CL1-5F4 cells. To verify the inhibitory effect of sulfasalazine on other tyrosine kinase activity, the phosphorylated levels of ErbB2/neu (pTyr^{1221/1222}) and EGFR protein (pTyr^{1086}) were also examined by Western blotting (Fig. 6D). At a concentration of 200 μmol/L for

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Sulfasalazine suppressed NF-κB activity, cell invasiveness, and AXL phosphorylation. **A,** effects of sulfasalazine and pI_{jBDN} on NF-κB reporter activities. CL1-5F4 cells were transfected with the NF-κB reporter plasmid, and after 1 d, cells were treated with sulfasalazine at the indicated dosages for 1 d before testing via the luciferase assay. Stable pI_{jBDN} vector transfectants of CL1-5F4 cells were also assayed for NF-κB reporter activity. *, *P < 0.05; **, *P < 0.01, compared with the parental CL1-5F4 control cells, Student’s paired t test. **B,** dose-dependent attenuation of cell invasiveness by sulfasalazine. pAXL-CL1-0 (clone 22) cells were assayed for 24-h invasiveness with or without various concentrations of sulfasalazine, pAXL-CL1-D (clone 22) cells were assayed for 24-h invasiveness with or without various concentrations of sulfasalazine. **C,** NF-κB–independent inhibition of AXL phosphorylation by sulfasalazine. pAXL-CL1-0 and pI_{jBDN}-CL1-5F4 cells were treated with 200 μmol/L sulfasalazine for the intervals indicated. Total AXL protein and p-AXL levels were assayed by Western blot analysis. **D,** ErbB2 and EGFR phosphorylation were suppressed by treatment with various concentrations of sulfasalazine for 60 min. Total ErbB2 and EGFR protein and the phosphorylated levels (pTyr^{1221/1222} of ErbB2/neu and pTyr^{1086} of EGFR protein) were assayed by Western blot analysis.
60 min, the phosphorylation of ErbB2/neu and EGFR protein was moderately inhibited by sulfasalazine, suggesting some nonselective effect of sulfasalazine on kinase activities of RTKs.

**Discussion**

Understanding of the roles of RTKs in cancer pathobiology has led to successful development of many therapeutic agents that specifically target RTKs (40). Our study has provided molecular genetic evidence that strongly correlates the expression of RTK AXL with invasiveness and drug resistance. This was done by selection of naturally occurring invasive and drug-resistant NSCLC clones and by establishing cell lines with overexpression and suppression of AXL protein. Ectopic overexpression of AXL was shown to increase the migration ability and the formation of protruding filopodia (Fig. 2B). Meanwhile, knockdown of AXL expression led to decreased migration (Fig. 2C). Comprehensive analyses of RTK expression profiles have revealed overexpression of AXL in clinical specimens and cell lines of NSCLC patients (13, 14). Shieh et al. (14) described the expression of AXL as statistically significant with respect to lymph node status and the patient’s clinical stage ($P < 0.0001$) in 58 patients. Our observation further strengthens the association of AXL expression and NSCLC metastasis.

The AXL subfamily members each possess two NH$_2$-terminal IG domains and two FN3 repeats in their extracellular regions (6). In the present study, using a series of truncated AXL mutants (Fig. 2D), we have shown that deletion of the first IG domain of AXL dramatically affects AXL-mediated cell invasiveness. Recently, Sasaki et al. (41) reported the crystal structure and mutational analysis of a minimal Gas6-AXL complex. The demonstrated high-affinity sites of Ghu56$^{AXL}$ and Ghu59$^{AXL}$ interacting with Gas6 are within this region (41). Our findings suggest that the Gas6-binding motif of the first IG domain is crucial for cell invasiveness. This assumption is further supported by our observation using small interfering RNA and the soluble AXL-extracellular decoy domain (AXL-Fc chimeras) that suppression of the level of Gas6 or AXL-Gas6 binding partially abolished the enhanced invasiveness of AXL-expressing CL1-0 cells (Supplementary Fig. S6A and B). The phosphorylation status of AXL was decreased in the Gas6 knockout cells and the AXL-Fc–treated cells as measured by immunoprecipitation/Western blotting (Supplementary Fig. S6C). Therefore, the expressed levels and binding activities of Gas6 seem to play a moderate role in enhanced cell invasiveness. Based on our observations, the intracellular tyrosine kinase domain, in contrast to the two FN3 domains, was also involved in the enhancement of cell invasiveness (Fig. 2D). Because AXL autophosphorylation, downstream signal transduction, as well as the oncogenic potential of AXL all lie within the tyrosine kinase domain (6, 27), enhanced cell invasiveness seems to be closely associated with kinase activity. In contrast to the role of AXL in cancer metastasis, the possibility of AXL playing a role in drug resistance remains to be determined. Although it has been well documented that AXL family-Gas6 signaling is involved in the inhibition of apoptosis mediated by various exogenous stimuli (6, 23, 25, 26), whether AXL plays a role in the prevention of apoptosis induced by chemotherapeutic drugs is still unknown. Another report showed the overexpression of AXL in a cisplatin-resistant ovarian carcinoma cell line (42). Our present observation is the first report showing a strong association between AXL expression and doxorubicin resistance in the drug-resistant CL1 variants and various cancer cell lines as shown by regression analysis (Fig. 3A and D; Supplementary Fig. S3). Because overexpression of AXL ectopically simultaneously conferred invasiveness and drug resistance (Figs. 2A and 3A and B) and because the drug-resistant variants concomitantly showed an increase in invasiveness and AXL expression (Fig. 3C and D), a link between AXL and the codevelopment of invasiveness and drug resistance may exist.

Further, based on our observations, resistance to doxorubicin was significantly elevated by AXL expression because doxorubicin, an inhibitor of DNA topoisomerase II, is also a well-known substrate of $\beta$-glycoprotein and other members of the ATP-binding cassette (ABC) family, and it remains to be clarified whether ABC genes or other genes are involved in AXL-related drug resistance. We have preliminarily observed that ABC proteins are not related with drug resistance and AXL expression in this CL1 series.6 We have observed previously that NF-κB is activated in cells challenged with various major anticancer drugs (38), and in the present study, we showed that NF-κB activation seemed to be the most important event for the development of drug resistance of the AXL–expressing cells in several ways. First, NF-κB activity was positively correlated with the levels of expression of AXL (Figs. 1D and 4B). Second, the ectopic overexpression of AXL invariably increased NF-κB activity and resistance to doxorubicin (Figs. 3A and B and 4A and B). Third, knockdown of AXL expression showed a concomitant decrease in NF-κB activity (Fig. 4A and B). Finally, treatment of cells with NF-κB inhibitors, sulfasalazine and Bay11-7082, as well as transfection with a dominant-negative IκB all synergistically diminished drug resistance of CL1-5F4 cells and the AXL-overexpressing CL1-0 cells (Fig. 5B; Supplementary Fig. S5). Additionally, elevated NF-κB activities, as examined by the luciferase reporter assay, were also shown in drug-resistant CL1-0/AdrR and CL1-0/VP16R cells (data not shown). Elevated NF-κB activity has been observed to be prevalent in lung cancer patients (43). In our previous studies, the suppression of NF-κB activity was found to increase the efficacy of chemotherapy (37, 38). In the current study, the disease-modifying anti-rheumatic drug, sulfasalazine, was found to dramatically reverse the drug resistance and invasiveness of NSCLC cells and concomitantly inhibited NF-κB activity and AXL phosphorylation (Figs. 5 and 6). What remains to be investigated is the mechanism underlying the suppression of cell invasiveness and drug resistance by sulfasalazine. Sulfasalazine is a cell-permeable nonsteroidal anti-inflammatory agent that specifically inhibits NF-κB–dependent transactivation through direct inhibition of IκB kinase (IκK-α and IκK-β) by antagonizing ATP binding (29, 30). In our study, sulfasalazine is more potent than PDTC and Bay11-7082 in inhibiting invasiveness and conferring synergism with doxorubicin (Fig. 5A and B), and this strong synergistic effect was dramatic and observed in all cell lines tested (Fig. 5B; Supplementary Fig. S5). Awasthi et al. (44) reported that clinically achievable concentrations of sulfasalazine modulate cisplatin resistance in two human SCLC lines. Reports by Arlt et al. (31, 33, 34) showed the use of sulfasalazine as a sensitizing agent in combination with VP16 or doxorubicin in pancreatic carcinoma cells. The spectrum and degree of possible synergistic effects induced by sulfasalazine in combination with traditional chemotherapeutic agents warrant further evaluation.

Targeted therapies are now emerging as an important strategy in the treatment of advanced NSCLC (4, 5). Thus far, several small-molecule RTK inhibitors have been developed as potential anticancer drugs (40). It is worth noting that overexpression of AXL in several cell lines has been found to result in receptor activation (41, 45). As shown herein, sulfasalazine inhibited not only NF-κB but also phosphorylation of AXL (Fig. 6C), and this inhibition...
of AXL phosphorylation was also observed in p85:BDN1-C1L-5/F4 cells, suggesting a NF-κB-independent mechanism of the sulfasalazine effects. In this respect, it is noteworthy that sulfasalazine was also found to augment cancer therapy in a NF-κB-independent manner (46, 47). Possible adverse effects induced by sulfasalazine on the enzymatic activity of RTK should be further evaluated because the expressed levels of p-ErbB2/neu and EGF-R were also inhibited, although moderately, by sulfasalazine at 200 μmol/L (Fig. 6D). The concentrations of sulfasalazine used in this study are clinically achievable (32, 47). The mechanism of the effects of sulfasalazine on AXL kinase activity needs to be further investigated. Taken together, our observations indicate that NF-κB signaling pathway is involved in the AXL-associated invasiveness and drug resistance, and the dual inhibition of NF-κB and AXL phosphorylation by sulfasalazine may provide an opportunity to effectively attenuate the invasiveness and drug resistance of NSCLC cells. For those refractory and/or metastatic cancers that express high levels of NF-κB and AXL, this synergism observed between sulfasalazine and doxorubicin and/or other chemotherapeutic agents is of enormous value and importance.

Acknowledgments
References
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